IDENTIFICATION AND FUNCTION OF

MALE MOOSE URINARY PHEROMONES

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By

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IDENTIFICATION AND FUNCTION OF MALE MOOSE URINARY PHEROMONES

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ABSTRACT

Olfactory communication and associated scent-marking activities play a major role in the behavioral ecology of many mammals. During the mating season (rut), scent marking associated with urine of male cervids is an important chemical cue to relay information to conspecifics. Specifically, adult male moose *(Alces alces)* dig rutting pits in which they urinate, and females respond strongly to urine deposited in pits.

A behavioral bioassay was developed to aid in the identification and function of adult male moose urinary pheromones, which elicited the behaviors observed in females during rut. Several behavioral bioassays were conducted to delineate the putative pheromones(s).

It was experimentally established that when female moose were presented with urine from the pre-rut and rut periods, females preferred the urine from rut. Moreover, this experiment documented that females responded markedly to constituent(s) in rut urine by wallowing. Rut urine can be chemically extracted and maintain its bioactivity when presented to female moose, the partition of the urine that had bioactivity was delineated. Information was provided on the chemical and physical nature of the chemosignal – not a protein, or carbohydrate, relatively non-polar, and of low molecular weight. Urinary constituents that may function as the putative pheromone(s) were characterized. Some of the chemical differences that existed in rut urine and may not function as chemical signals were eliminated. Also provided, was evidence that female moose may utilize the main olfactory system to detect chemosignals present in rut urine.

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CHAPTER 1

GENERAL INTRODUCTION

1.1 Reproductive Biology of Moose

Moose *(Alces alces)* are the largest member of the deer family (Cervidae). Like most members of Cervidae, moose exhibit sexual dimorphism. Adult males may weigh > 770 kg (Schwartz et al., 1987), and maintain palmate antlers that are cast and regrown each year (Chapman and Feldhamer, 1990). Adult females may obtain weights > 570 kg (Schwartz et al., 1987). Like many other ungulates, moose are polygynous, and males make no parental investment in young. During most of the year, males and females spatially segregate (Miquelle et al., 1992; Bowyer et al. 2001). Nonetheless, during the mating season (rut), male and female moose aggregate in specific areas where both scent-mark (Bowyer et al., 1994).

Females may become sexually mature at approximately 16 months old, and begin their estrous cycle in the late summer. Schwartz and Hundertmark (1993) determined that the estrous cycle of females lasted between 22 to 28 days. During the estrous cycle, the ovaries produce increased quantities of progesterone and estrogen (Stewart et al., 1985; Monfort et al., 1993) that enhances the development of the vagina, oviducts, and the bicornate uterus. Ovulation is closely associated with estrus when large amounts of estrogen enter the blood system. During estrus the female is receptive and will allow mounting by males. The period of estrus in females varies greatly, but typically lasts for 15 to 26 hrs (Schwartz and Hundertmark, 1993).

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Like most deer species, female moose are polyestrous (Edwards and Ritcey, 1958; Markgren, 1969). If the female does not become pregnant after mating, the estrous cycle begins again. If unbred, captive females may experience as many as six recurrent estrous cycles during the mating season (late-September through late-March) (Schwartz and Hundertmark, 1993). Nonetheless, two to three estrous cycles are more typical for wild female moose (Schwartz and Hundertmark, 1993).

The gestation period lasts approximately 8 months, with a range of 216 to 246 days (Markgren, 1969; Verme, 1970; Stewart et al., 1987; Schwartz and Hundertmark, 1993). Parturition is extremely synchronized in moose. Bowyer et al. (1998) reported that the median birth of date for moose in interior Alaska was 25 May. In many species, late-born young are at a selective disadvantage (Clutton-Brock et al., 1987); therefore, females with offspring conceived earlier in the mating season may benefit via enhanced survivorship of those young (Keech et al., 2000). For moose at high latitudes, timing parturition early during a relatively short growing season may be necessary to obtain requirements of both mother and young to survive harsh winter conditions (Bowyer et al., 1998; Bowyer et al., 1999; Keech et al., 2000).

Typically, female moose give birth to either a single young or twins (Franzmann and Schwartz, 1998). Occasionally, females give birth to triplets (Peterson, 1955; Franzmann and Schwartz, 1985; Bowyer et al., 1998), and the possibility of quadruplets exists (Martin, 1989). In female moose, senescence occurs from about 12 years of age onward (Ericsson et al., 2001).

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Bubenik and Timmermann (1982) noted that males in their prime (ages 6 to 9) produced more sperm earlier in the mating season than did yearlings. Males become sexually mature at 16 months old and are capable of impregnating females (Schwartz et al., 1982). Nonetheless, yearling males are usually excluded from active mating by more mature, dominant males (Lent, 1974; Knowles, 1984; Bubenik 1987; Miquelle, 1993; Van Ballenberghe and Miquelle, 1996; Stewart et al., 2000; Bowyer et al., 2001a).

Testosterone produced by the testes plays a dual role in the physiology of males. One function is the development of secondary sex characteristics (e.g., antlers). Secondly, testosterone is important in the maintenance and expression of the reproductive system including male sexual behavior. Testosterone reaches its maximum level just before the peak of rut in late September and early October (Franzmann and Schwartz, 1998). Levels of testosterone may explain the decrease in appetite and concomitant weight loss observed in males during the rut. Franzmann et al. (1978) and Schwartz et al. (1987) noted that adult male moose can lose from 12 to 19% of their prerut body mass.

Moose are a polygynous and nonterritorial species (Miquelle, 1990). Similar to white-tailed deer (*Odocoileus virginianus*), moose throughout most of North America exhibit a tending-bond mating system - the dominant male defends an estrous female until she mates with him, and then moves on in search of additional mates (Altmann, 1959; Geist, 1963). Bowyer et al. (2003) noted that Alaskan moose exhibit a harem mating system similar to elk *(Cervus elaphus)* - a dominant male herds and defends a group of females without regard to their estrous state and does not permit sexually mature males into his rutting group. Rutting groups may range in size to 30 or more adults

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(Bowyer et al., 2003). In Alaska, a second rut occurs in late October and early November, and the mating system reverts to a tending bond. Moreover, males do not dig rutting pits during the second rut. Bowyer et al. (1994) hypothesized that scent marking of trees by males served a similar function to rutting pits thereby attracting to females that were not successfully bred during their first rut.

During rut, males engage in ritualized fighting that may include protracted displays and charges. Van Ballengberghe and Miquelle (1993, 1996) noted that this behavior occurred mostly before and after the peak of rut. These sparring matches are of low intensity, and antler positioning and pushing are done gently. In contrast, serious fighting that occasionally may result in death occurs around the peak of rut. Additionally, aggressive behavior among females also occurs during the rut.

In cervids, scent-urination during the mating season has been well documented (Espmark, 1964; Mossing and Damber, 1981; Kojola, 1991). Some cervids such as elk, fallow deer *(Dama dama)* and moose, dig rutting pits or wallows in which urine is deposited (McCullough, 1969; Bowyer and Kitchen, 1987; Miquelle, 1991; Massei and Bowyer, 1999). During rut, male moose attract females by scent marking with their urine. Adult males initiate pit digging early in the mating season by creating a shallow depression (pit) in the ground by digging with their forefeet. This digging is combined with urinating on the disturbed soil (Lent, 1974; Miquelle, 1991; Van Ballenberghe and Miquelle 1993). Urine excreted during rut has a unique strong smell. These males impregnate their pelage with the scent by splashing urine and mud onto themselves using their front hooves, slapping urine with the underside of their antlers, and lying

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(wallowing) in the pit (Miquelle, 1991). Females also wallow in these pits and impregnate their pelage with urine deposited by adult males (Miquelle and Van Ballenberghe, 1985).

Franzmann and Schwartz (2000) proposed that female moose might exercise some choice in selecting a male. Aggressive interactions directed towards males by females are not uncommon. Indeed, when a subdominant male approaches a female, she may warn the dominant male of the interloper by a protest vocalization (Van Ballenberghe and Miquelle, 1996).

Most mating occurs during autumn, with the peak of rutting activity occurring between late-September and early-October (Franzmann and Schwartz, 1998; Bowyer et al., 2003). If mating is unsuccessful during the first estrus that coincides with peak of rut, successive estrous cycles may occur at 20 to 30-day intervals (Edwards and Ritcey, 1958; Markgren, 1969). Schwartz and Hundertmark (1993) estimated that 88, 11, and 1% of the conceptions occurred during the first, second, and third estrous periods, respectively.

Conception during later estrous periods results in parturitions in summer that may be disadvantageous to young because of a shortened time for growth during summer, and the consequent small size of young at the beginning of winter (Keech et al., 2000). Additionally, late-born young experience higher rates of mortality compared with others in their cohort (Keech et al., 2000). Late parturitions occur in Sweden (Markgren, 1969) but are limited for moose in North American (Coady, 1974). For moose at high latitudes, studies have demonstrated that second-estrous females shortened the length of gestation

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markedly to have sufficient time during the short summer season to provision offspring (Rachlow and Bowyer, 1991; Berger, 1992; Schwartz and Hundertmark, 1993).

The bond between females and their young normally last from birth to shortly prior to parturition the following year. Typically, the female drives her offspring (by then a yearling) away in preparation for the birthing of a neonate. If females do not give birth or the neonate does not survive, young from the previous year may remain with the female during its second year (Chapman and Feldhamer, 1990; Molvar, 1993).

1.2 The Olfactory Systems

Olfaction is one of the principal sensory modalities for many animals, and chemosensory communication is especially well developed in most mammals. Indeed, the mammalian olfactory system has the ability to detect and distinguish thousands of compounds that provide important information about the environment (Stowers, et al., 2002; Trinh and Storm, 2003). In aquatic and terrestrial environments, olfactory perception begins when volatile compounds and odorants approach specific receptors located on sensory neurons. Chemosignals are processed by two distinct olfactory systems: the main olfactory system (MOS), and the accessory olfactory system (AOS) (Dulac and Torello, 2003). Furthermore, each system has its unique pathway to different regions in the brain (Halpern, 1987).

The accessory olfactory system is believed to be involved mainly with matters related to reproduction (Halpern and Kubie, 1984; Halpern, 1987; Wysocki, 1989; Dulac and Torello, 2003). The AOS processes volatile and nonvolatile chemical cues (Meredith, 1991; Rasmussen et al., 1997). Furthermore, the receptor organ (vomeronasal

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organ) for the AOS is involved in processing chemosignals that in many mammals may be linked to reproduction. Indeed, the neurons associated with the vomeronasal organ project directly to the accessory olfactory bulb of the AOS (Kevetter and Winans, 1981; Halpern, 1987).

In contrast to the AOS, the MOS is believed to serve a more generalized function for detection and discrimination of a vast number of stimuli in the environment. Nevertheless, the MOS processes volatile chemical cues thought to be important in social interactions and reproduction (Jacob and McClintock, 2000; Whittle et al., 2000; Beauchamp and Yamazaki, 2003; Preti et al., 2003). Moreover, recent studies have demonstrated that nonvolatile chemosignals (e.g., proteins), which may ultimately be processed by the MOS, are important in reproduction (Novotny, 2003; Rasmussen et al., 2003).

There may be a convergence between the MOS and the AOS that allows chemical signals to be detected by both systems (Sipos et al., 1995; Meredith, 1991; Trinh and Stern, 2003). Indeed, studies have proposed that both systems are involved in pheromone reception and bioactivity (Rosell and Sundsdal, 2001; Sam et al., 2001). A pheromone is a substance secreted or excreted into the environment by one individual that, on being received by a conspecific, elicits a definitive behavioral, developmental, or endocrine response (Karlson and Lüscher, 1959).

Four different classes of pheromones (Table 1.1) are recognized: 1) modulator; 2) releaser; 3) signaling; 4) and primer.

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Table 1.1 Classes of Pheromones

CLASS OF PHEROMONE	SPECIES	REFERENCE
Modulator: Changes how individuals behave or react to a current situation by influencing psychological state. Provides information about presence, condition, or status.	Human *(Homo sapiens)*	Jacob and McClintock (2000)
Releaser: Causes specific, well-defined responses that include overt displays of attraction and copulation. Operates quickly.	Domestic pig *(Sus scrofus)*	Melrose et al. (1971)
Signaling: Operates in an intermediate or immediate time course and promotes less specific/stereotyped behaviors. Involves individual/kin recognition.	Asian elephant *(Elaphus maximus)*;	Rasmussen and Greenwood (2003);
	Human	Wysocki and Preti (2004)
Priming: Results in changes to the receiver's, behavior, physiological, and hormone state. Behavioral responses to the pheromone(s) may be overt (e.g., mouse). Changes in the neuroendocrine system may operate over days or weeks.	House mouse *(Mus domesticus)*;	Meredith (1991); Novotny et al. (1999);
	Human	Preti, et al. (2003); Alport (2004)

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1.3 Mammalian Olfactory Communication Using Chemical Signals

Mammals use an array of physical, social, and environmental factors to coordinate and synchronize reproduction. Pheromones and other chemical signals are important in intra- and interspecific identification (Müeller-Schwarze et al., 1984; Passanisi and MacDonald, 1990; Thurber et al., 1992; Lewis and Murray, 1993; Adams et al., 2003), territorial marking (Gilbert, 1973; Kitchen, 1974; Gosling, 1987; Johansson et al., 1996; Moorcroft, 1999; Massei and Bowyer, 1999; Kimura, 2001), alarm systems (Müeller-Schwarze, 1969; Booth and Signoret, 1992; Kiyokawa et al., 2004), predator-prey relationships (Regnier and Wilson, 1971; Mattina et al., 1991; Young, 1993), maternal-infant bonds (Poindron et al., 1988; Poran et al., 1993), social structure and organization (Passanisi and MacDonald, 1990), and sexual behavior (Alteri and Müeller-Schwarze, 1980; Rasmussen et al., 1986; Rasmussen, 1988; Menzies et al., 1992; Rasmussen et al., 1997; Iwata et al., 2000).

Ungulates exhibit a diverse array of scent-marking behaviors (Coblentz, 1976; Leuthold, 1977; Benner and Bowyer, 1988; Gosling, 1987; Bowyer et al., 1994; Oehler et al., 1995). Moreover, considerable evidence links olfaction and reproduction among ungulates (Watson and Radford, 1960; Fraser, 1968; Coblentz, 1976; Bakke and Figenschou, 1990; Miquelle, 1991; Booth and Signoret, 1992; Cohen-Tannoudji et al., 1994; Hamada et al., 1996; Ma and Klemm, 1997; Iwata et al., 2000). Studies on domestic pigs and sheep have provided detailed information on olfactory mechanisms in ungulate reproduction. Pheromones of those two species have been identified and related to specific behavioral and physiological changes (Booth and Signoret, 1992; Dorries et

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al., 1997). Indeed, the role of chemical communication in the reproductive behavior of ungulates, especially the behaviors associated with scent marking by males has been studied for many species (Coblentz, 1976; Booth and Signoret, 1992).

In male cervids, scent marking likely functions in several areas, such as reproduction and the establishment of hierarchies (Miquelle, 1991). Species such as North American elk, fallow deer, and moose scent-mark by urination (scent-*urination*) during the mating season (Bowyer and Kitchen, 1987; Miquelle, 1991; Massei and Bowyer, 1999). Scent-urination in adult male moose is likely directed more towards females than males because that behavior is not temporally correlated with aggressive interactions between males, and female moose are strongly attracted to the unique smell of the urine excreted by rutting males (Miquelle, 1991).

During rut, adult males can lose from 12 to 19% of their pre-rut body weight (Franzmann et al., 1978; Schwartz et al., 1987). Whittle, et al. (2000) proposed that the catabolism of endogenous reserves as a result of decreased food intake coupled with changes in androgen levels probably account for the unique odor of urine during the rut. Mammalian chemosignals are typically entrenched in a very complex biological matrix such as glandular secretions or urine. Indeed, urine contains a concentrated source of volatiles that are deposited in the environment upon excretion (Albone, 1984). Studies on solvent-extracted urine samples indicated differences in the chemical composition of urine from adult male moose during rut compared with nonrutting adult males (Whittle et al., 2000).

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Moose evolved in dense boreal forests where environmental constraints on visual, and auditory communication have lead to the development of exceptional olfactory capabilities (Clifford and Witmer, 2004). Indeed, olfactory communication may be a critical component in the reproductive biology of moose (Miquelle and Van Ballenberghe, 1985; Miquelle, 1991; Van Ballenberghe and Miquelle, 1993; Schwartz and Hundertmark, 1993; Van Ballenberghe and Miquelle, 1996; Bowyer et al., 1998; Whittle et al., 2000).

1.4 Overview, and Goals of Thesis

Chemical differences exist in urine from female and male moose (Whittle, 1999). Additionally, there are qualitative and quantitative differences between urine of adult male moose during the rut and nonrut periods (Whittle et al., 2000). Therefore, I wanted to: 1) develop a behavioral bioassay that would aid the identification of urinary constituents which elicited the behaviors observed in females during the rut, 2) develop and utilize a variety of analytical techniques and commercially available standards to identify putative pheromones, and 3) provide empirical evidence that female moose use the MOS to detect chemosignals retained in the urine of adult males.

No studies have demonstrated that female behaviors in response to urine from the rut are not elicited also by urine from periods outside of rut. To make inferences regarding the bioactivity of urinary constituents, it was necessary to establish behaviorally that female moose preferred the urine from the rut in comparison with nonrut periods. This crucial test would establish the baseline of female behaviors for all behavioral bioassays.

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Urine from adult male moose during the rut is a complex mixture of over 100 compounds (Whittle, 1999). To determine which urinary constituents elicited a female response, I used a variety of chemical techniques to delineate which partition of the urine had bioactivity. Therefore, it was imperative that I established that bioactive constituents can be retained after undergoing the rigors of chemical techniques, and could be characterized using analytical tools (e.g., gas chromatography/mass spectrometry (GC/MS)).

Because of the complex nature of nonrut and rut urine, I established that any chemical differences observed were not artifacts. Furthermore, I eliminated some of the chemical differences that existed in rut urine that may not function as chemical signals that do not elicit behavioral responses in females.

The overall objective of this study was to enhance the knowledge of the reproductive biology of moose. Specific hypotheses are presented in each section of the behavioral bioassays, and experiments conducted.

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CHAPTER 2

GENERAL EXPERIMENTAL PROCEDURES

Urine samples (≥700 ml/animal) were collected from 3 adult male moose held in captivity at the Kenai Moose Research Center, a facility operated by the Alaska Department of Fish and Game, on the Kenai Peninsula, Alaska, USA (60°N, 150°W). Urine was collected by holding a 1-l bottle affixed on a 1.8-m pole while an animal urinated. Samples from 3 adult males consisted of urine from pre-rut (early September), rut (late September-early October), and post-rut (late-March). Samples were stored in a freezer at –70°C until needed for chemical analysis, or presentation to female moose during bioassays. Frozen samples were thawed in a refrigerator at 10° C. Prior to handling (e.g., extraction), the pH of the urine was determined using a Fischer Scientific, Model 10 electronic pH meter.

Urine samples were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a 5% phenyl methyl siloxane capillary column (0.25-μm x 30-m; Alltech Inc.), interfaced with a HP 5972 mass selective detector (GC/MS). The injector port was set at 275°C. The initial oven temperature was maintained at 50°C for 3 min and then increased to 300°C at 4°C/min. Data acquisition and operating parameters for the mass spectrometer were set as follows: scan rate .313 s from *m/z* 40 to 400; ion source temperature set to 280°C, with electron energy at 70 eV. Samples (1μl) were injected into a splitless injector and eluted using a flow rate of 1.0 ml/min. Initial peak identification was made through the use of a library of standard mass spectra (Wiley138),

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and subsequent identification was accomplished by matching retention times and mass spectra with authentic samples.

All standards and solvents used were purchased from Sigma/Aldrich and used without further purification. Silica gel (40μm) for flash chromatography was purchased from VWR.

Behavioral bioassays of female moose occurred during rut (late September through early October). Females were allowed to acclimatize in their holding area for 12-24 hrs before bioassays began. All animals used for this study were provided by the Alaska Department of Fish and Game – Moose Research Center, and were greater than 1 year old. For presentation to female moose, randomized samples of control, urine, urine subjected to chemical fractionation, or synthetic compounds were applied to a 3 x 3 cm commercial sponge that was affixed to the inside of a bird feeder suet basket at a height of 2 m, with a minimum distance of 0.5 m between samples. Sample and control amounts used in bioassays were 5 ml, or its equivalent. To avoid contamination, baskets were individualized for each sample (e.g., baskets used for the sample were not used for the control sample). Additionally, disposable rubber gloves were used during sample placement and handling, and were changed between each trial. During 10-min trials, continuous and categorical behavioral data were collected (Altmann, 1974). Unless indicated otherwise, all behavioral trials were replicated a minimum of 3 times. Table 2.1 illustrates the typical behaviors that were recorded during bioassays. These procedures were approved by an Institutional Animal Care and Use Committee at the University of Alaska Fairbanks, and the Alaska Department of Fish and Game.

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Statistical analyses were performed with ANOVA for Excel 2000, and SAS Version 9.1. For Section 2.1, Fischer exact probability test was performed using SPSS for Windows. Comfort behaviors (termed: 'Neutral') were eliminated from the statistical analyses based on the following rationale: 1) During the rut, non-captive female moose engage in Neutral behaviors before, and after access to rutting pits; and 2) This study investigated the function, and identification of male moose urinary pheromones; therefore, pertinent data for this investigation was the response of females to that stimuli (e.g., sample), and the control.

Table 2.1. Typical Behaviors of Female Moose Recorded During Behavioral Bioassays

Smell sample	Smell and lick at sample
Smell and rub near sample	Paw at ground or wallow
Standing	Walking
Aggressive interactions	Drinking
Urinate	Bedding

2.1 Test of Bioactivity of Pre-rut Versus Rut Urine

(Bioassays conducted in Autumn 2004)

The purpose of this bioassay was to determine if female moose preferred the urine from the rut period to that of the pre-rut. Data collected from these studies served as the baseline for all behavioral bioassays previously conducted. Bioassays were conducted on 5 adult female moose. Treatments for the bioassay trials consisted of rut and pre-rut urine. Trials were replicated 4 times.

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2.2 Headspace Analysis of Major Volatiles in Pre-rut, Rut, and Post-rut Urine

Urine (25 ml) from each period (pre-rut, rut, and post-rut) was equilibrated to room temperature in a 60 ml vial sealed with a septum. The headspace was sampled for 30 min at room temperature using a Solid Phase Microextraction (SPME) apparatus (Supelco) equipped with a StableFlex divinylbenzene/carboxen/poly-dimethylsiloxane fiber. Prior to each sampling, the fiber was preconditioned in the injection port of the GC/MS for 10 minutes at 275°C.

Gas Chromatography/Mass Spectrometry of Headspace Volatiles After adsorption, the fiber was introduced into the injector of a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a 5% phenyl methyl siloxane capillary using the temperature program as noted in the General Experimental Procedures section.

Chromatographic peaks that had abundance greater than 4% of total TIC were considered major volatile peaks. These data were used in the statistical analyses.

2.3 Test of Bioactivity of Pentane Extracted Rut Urine
(Bioassays conducted in Autumn 1999)

Organic compounds were obtained by extracting 220 ml of rut urine with three successive extractions of 100 ml pentane, dichloromethane, and ethyl acetate. The organic layers were then dried (Na_2SO_4). To remove the emulsion produced during the pentane extraction, the organic layer was filtered through glass wool. Using the same protocols described previously, deionized water was successively extracted with the three solvents, but filtration using glass wool was not necessary. The behavioral response of five female moose when presented with samples from the pentane, or dichloromethane, or ethyl acetate extracts, along with the control sample was recorded.

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2.4 Test of Bioactivity of 2 Major Urinary Compounds: *p*-Cresol and Geraniol
(Bioassays conducted in Autumn 2000)

Experiment 1: The pH of post-rut urine samples was adjusted to that of rut urine using a 10% solution of H_3PO_4, and a saturated solution of $NaHCO_3$. Treatments for Experiment 1 bioassay trials consisted of pH-adjusted post-rut, and post-rut urine (negative control).

Experiment 2: Treatments for Experiment 2 bioassay trials consisted of pH-adjusted post-rut, and rut urine (positive control).

Experiment 3: The headspace analyses allowed me to obtain the relative concentration of *p*-cresol, and geraniol present in rut urine. These were determined by comparing their integrated peak area from GC/MS total ion chromatographs, which resulted from headspace analyses. Accordingly, commercial (Aldrich, Inc.) samples of *p*-cresol and geraniol corresponding to the concentration found in rut urine were added to the pH-adjusted post-rut (Cocktail). Treatments for Experiment 3 bioassay trials consisted of pH-adjusted post-rut (negative control), and the Cocktail.

Experiment 4: Treatments for Experiment 4 consisted of the Cocktail, and rut urine (positive control).

Bioassays were conducted on 4 female moose. Experiments 1, 2, and 3 were not replicated for individuals. Experiment 4 was replicated 2 times for each female.

2.5 Test of Bioactivity of 3 Unique Preparatory Gas Chromatography Effluents
(Bioassays conducted in Autumn 2001)

Pentane-soluble organic compounds were obtained by extracting 240 ml of rut urine with three successive extractions of 150 ml of pentane. To remove the emulsion

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produced during the extraction process, the organic layer was placed in a sealed Teflon tube and centrifuged in a Dupont, Model Sorvall RC 5B Plus centrifuge for 10 min at 5000 RPM at a temperature of 4°C.

One-half ml of concentrated sample was obtained by rotoevaporation of 50 ml of extract at 30°C. One hundred µl of the concentrated sample was injected into a Gow Mac Series 150 chromatographic unit (GC prep) equipped with a 2.1-m x 0.63 cm stainless steel column packed with 15% OV-1 on Chrom-P. A flow rate of 55 ml/min, an injection temperature of 150°, and a constant oven temperature of 150°C were maintained. To minimize premature condensation, the outlet port had a length of 3.5 cm that was heated externally with a heat gun to maintain a temperature of less than 200°C. Effluent was collected in a U-shaped glass collection tubes that were cooled in liquid nitrogen. To minimize effluent loss from collection tubes, a swab of glass wool, and 0.25 ml of pentane was added to the tubes prior to the initial injection of concentrated sample. The collection tubes were changed every 10 minutes to yield 3 fractions over a 30-minute period. The time required to change collection tubes was ≤8 sec. Collection tubes were kept cold in liquid nitrogen until the sample was transferred to storage vials. Collection tubes containing the effluents (fractions and control) were kept cold in liquid nitrogen, rinsed with 1-ml pentane to collect any sample that may have adhered to walls of the tube, and then transferred to storage vials. Samples were stored in a freezer at -50°C.

Fraction 1 consisted of effluent collected during the first 10 minutes, Fraction 2 consisted of the effluent collected over the second 10 minutes, and Fraction 3 consisted of the effluent collected over the third 10 minutes. Additionally, a second injection was

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made during which the entire effluent was collected over the entire 30-minute period; this was termed the Whole fraction. To serve as the control, 100 µl of pentane was injected and the effluent was collected for 30 minutes.

To maintain the integrity of each of the 3 fractions, and the Whole fraction that was collected, the column was washed with injections of pentane in between sample injection. Accordingly, after each sample injection, 200 µl of pentane was injected and was allowed to off-gas for 5 min. This interval was followed by another 200 µl injection of pentane, which was allowed to off-gas for 10 min. The pooled effluents of each of the 3 fractions, and the Whole fraction were then diluted with pentane to yield the 5 ml aliquot that was applied to the bioassay sponges.

Behavioral bioassays were conducted on 7 female moose. Treatments for the bioassay trials consisted of 4 samples (Fraction 1, Fraction 2, Fraction 3, and the Whole fraction). The effluent collected when pentane was injected into the chromatographic unit served as the control.

2.6 **Test of Bioactivity of 3α-Hydroxy-5β-androstan-17-one (steroid), and Flash Chromatography Eluants** (Bioassays conducted in Autumn 2002)

3α-Hydroxy-5β-androstan-17-one:

Experiment 1: The relative concentration of 5α-hydroxy-5β-androstan-17-one (steroid) in crude rut urine was about 0.25 mg/ml as determined by gas chromatographic comparison of peak area with a standard sample. Post-rut urine was adjusted to the pH of rut urine using a 10% solution of H_3PO_4, and the appropriate amount of steroid was added to obtain a concentration 0.25 mg/ml.

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Experiment 1a: The steroid concentration was increased 75 times from that of Experiment 1 (0.25 mg/ml). To test for the efficacy, it is not uncommon to increase synthetic putative pheromone concentrations beyond that found in the actual sample (Rasmussen et al., 1997). Accordingly, the appropriate amount of steroid was added to the pH adjusted post-rut urine to obtain a concentration of 18 mg/ml.

Experiment 2: *p*-cresol, at a previously determined concentration in rut urine, was added to the pH adjusted post-rut urine, along with the steroid at a concentration of 18 mg/ml.

Experiment 3: The steroid was added to pH adjusted post-rut urine to yield a concentration of 18 mg/ml.

Experiment 4: The steroid was dissolved in methanol to yield a concentration of 18 mg/ml.

Five female moose were used in the bioassays for Experiments 1, 3, and 4. For Experiments 1a, and 2, three females were used. Only Experiments 1a, and 4 were replicated for individual moose. The control used for Experiments 1, 1a, and 2 was post-rut urine. For Experiment 3, rut urine served as the positive control. Methanol was used as the negative control in Experiment 4. Behavioral data were collected during 10-min trials.

Flash Chromatography: Organic compounds were obtained by extracting 900 ml of rut urine with 3 successive extractions of 500 ml of pentane. To remove the emulsion produced during the extraction process, the organic layer was placed in sealed Teflon tube and centrifuged in a Dupont, Model Sorvall RC 5B Plus centrifuge for 10 min at

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5000 RPM at a temperature of 4°C. Rotoevaporation at 30° C of 1000 ml extract yielded 10 ml of concentrated sample. The concentrated sample was flash chromatographed on silica gel using a 40 mm diameter flash column (VWR).

Three solvent compositions of chloroform and methanol (99:1, 97:3, and 95:5) were used to separate urinary components. In total, 30 fractions were collected, and based on thin-layer chromatography (TLC) analyses, were combined into 3 samples/experiments (Table 2.2).

Using a chloroform and methanol (99:1) solvent, I collected fractions 13 through 17. With the second solvent system of chloroform and methanol (97:3), fractions 1 and 2 were collected. These fractions were pooled, and used for Experiment 5.

The second solvent system consisted of chloroform and methanol (97:3). Fractions 3 through 11 were collected and served as Experiment 6.

The third solvent system consisted of chloroform and methanol (95:5). Fractions 1 through 7 were collected and used in Experiment 7.

The fractions that served as the control samples were also obtained by flash chromatography using the three same solvent systems, and fraction collection scheme.

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Table 2.2 Flash Chromatography Fractions Presented to Female Moose

Solvent System/ Experiment	A Chloroform:Methanol 99:1	B Chloroform:Methanol 97:3	C Chloroform:Methanol 97:5
Experiment 5	Fractions A13-17	Fractions B1-2	----
Experiment 6	----	Fractions B3-11	----
Experiment 7	----	Fractions B12-18	Fractions C1-7

Five female moose were used for the behavioral bioassays. Behavioral data were collected during 10-min trials.

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CHAPTER 3

RESULTS

3.1 Test of Bioactivity of Pre-rut Versus Rut Urine

Female moose spent significantly more time investigating the rut than the pre-rut sample ($F = 136.94$, d.f. = 1, 48, $P = 0.0001$) (Figure 3.1). Mean time spent investigating the rut sample was 273 seconds (SD±106), which comprised 92% of the trials. In contrast, the mean time that females spent investigating the pre-rut sample was 24 seconds (SD±13), which comprised 8% of the trials. Additionally, only the rut sample elicited wallowing behaviors ($p<0.000001$).

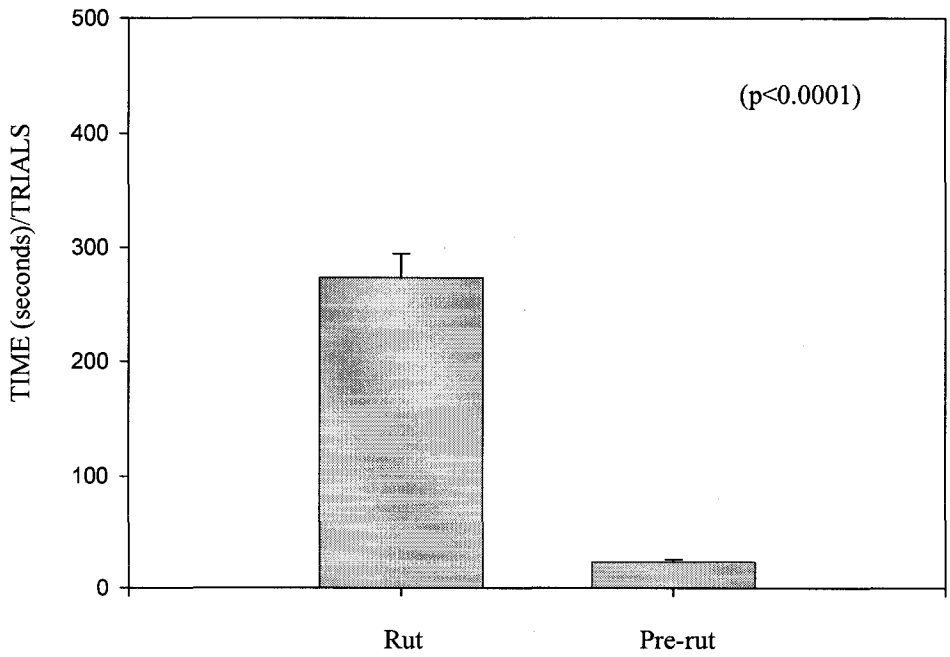

Figure 3.1 Female response to pre-rut and rut urine (n=5). Mean time spent (±SE) investigating pre-rut and rut urine during 10-minute replicated trials.

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3.2 Microextraction (SPME) Headspace Analysis

Headspace analysis of pre-rut, rut, and post-rut urine indicated that there were significantly ($F = 26.44$, d.f. = 2, 24, $P = 0.00008$) more volatile compounds detected in the headspace above rut urine than from either pre-rut, or post-rut urine samples (Figure 3.2). The mean number of volatile compounds with a peak abundance of 4% of the total ion chromatogram for pre-rut, rut, and post-rut was 7 (SD±9), 34 (SD±11), and 8 (SD±6), respectively (Figure 3.3).

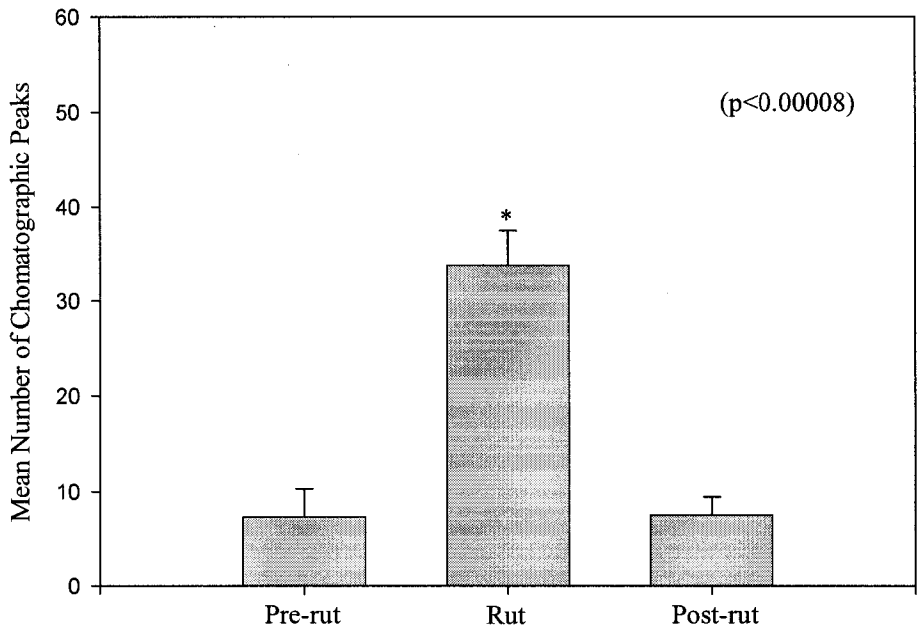

Figure 3.2 Mean number of volatile urinary compounds (±SE) with 4% of total TIC from the urine of adult male moose (n=3) for the pre-rut, rut, and post-rut periods detected using SPME headspace analyses coupled with GC/MS.

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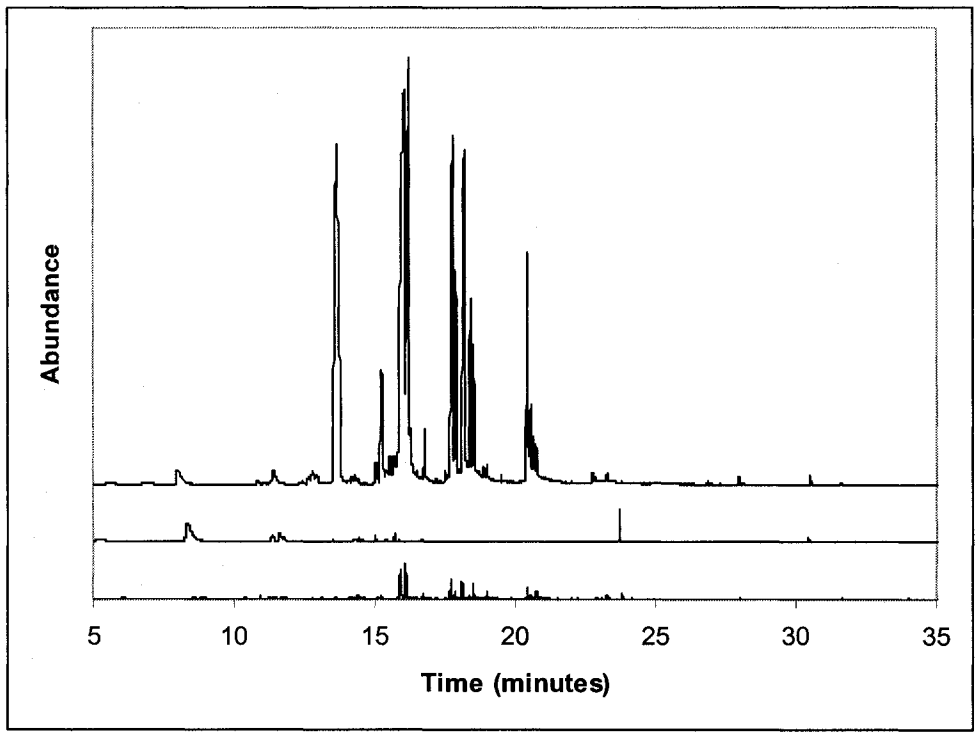

Figure 3.3 Chromatograph of volatile compounds detected by SPME headspace analyses of pre-rut, rut, and post-rut urine samples. The upper trace represents the volatile profile of rut urine, the middle trace represents the volatile profile of pre-rut urine, and the lower trace represents the volatile profile of post-rut urine from 3 adult male moose.

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3.3 Test of Bioactivity of Pentane Extracted Rut Urine

During 10-minute trials, female moose spent on average 416, 319, and 326 seconds, investigating the pentane, dichloromethane, and ethyl acetate urine extracts, respectively. There was no significant difference ($F = 2.02$, d.f. = 2, 42, $P = 0.14$) in the mean time females spent investigating the 3 extracts (Figures 3.4 and 3.5).

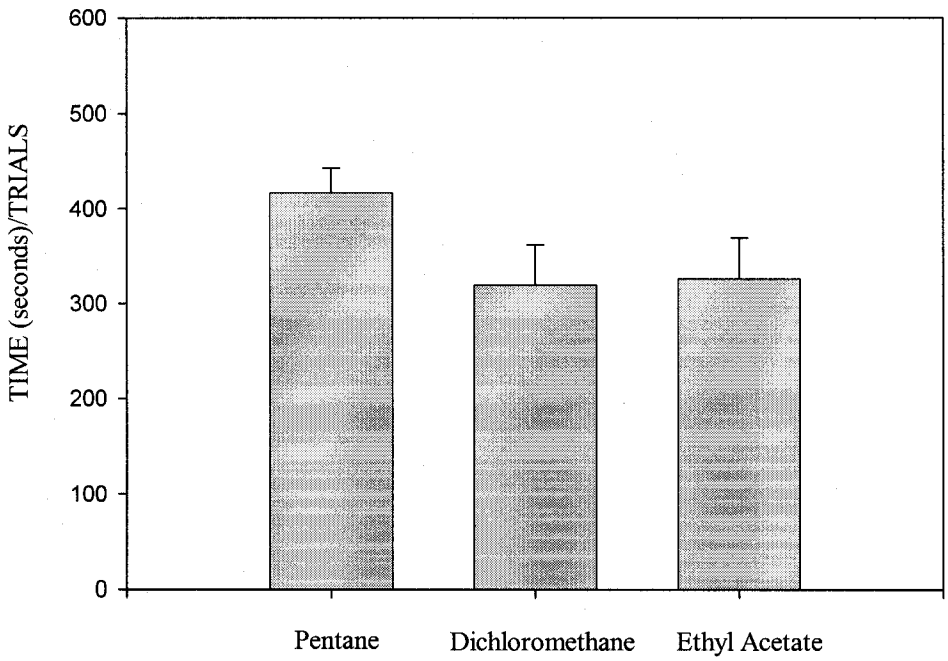

Figure 3.4 Female response to 3 rut urine extracts (n=5). Mean time spent investigating 3 rut urine extracts during 10-minute replicated trials.

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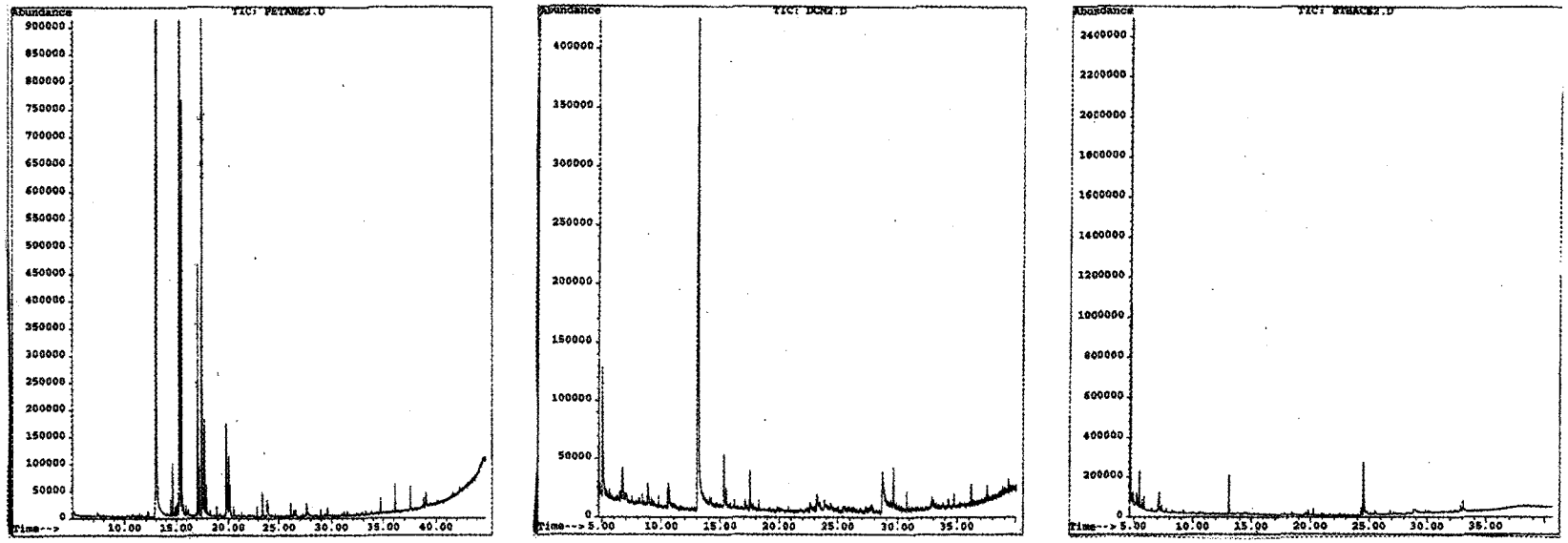

Pentane Extract

Dichloromethane Extract

Ethyl Acetate Extract

Figure 3.5 Chromatographs of pentane, dichloromethane, and ethyl acetate extracted rut urine

There was, however, a significant difference (F = 15.33, d.f. = 2, 24, P = 0.0005) in the time female moose spent in wallowing behaviors for the 3 extracts. Females engaged in wallowing behaviors in response to the pentane extract for a total of 153 seconds (SD ± 11), or 1.7% of the trials. In comparison, females engaged in wallowing behaviors in response to the dichloromethane, and ethyl acetate extracts for 14 seconds (SD ± 3), or 0.76%, and 4 seconds (SD ± 1) or, 0.44% of the trials, respectively (Figure 3.6).

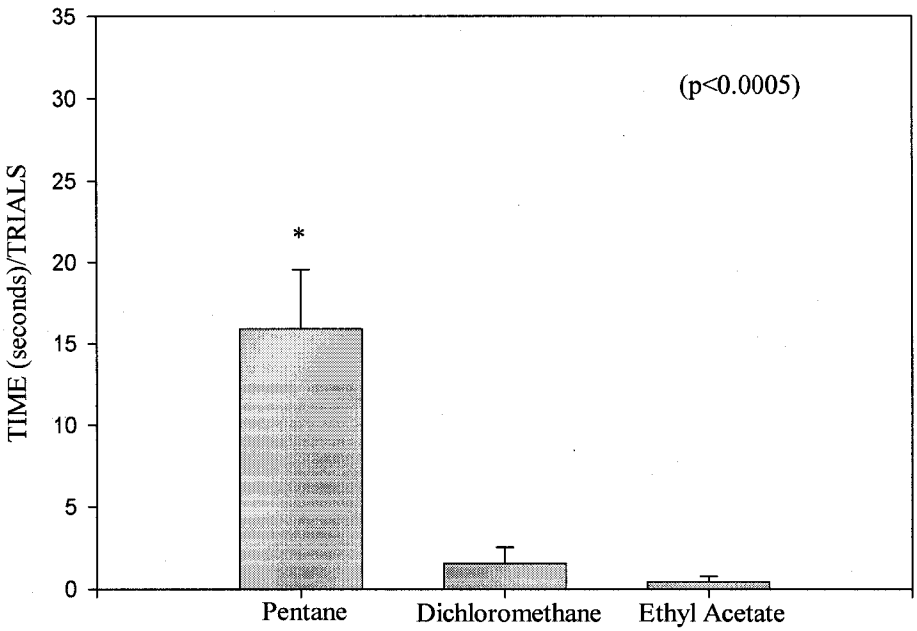

Figure 3.6 Mean time (±SE) spent by female moose (n=5) in wallowing behaviors in response to 3 rut urine extracts during 10-minute replicated trials.

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3.4 Test of Bioactivity of *p*-Cresol, and Geraniol (Cocktail)

Experiment 1 tested the response of females to pH-adjusted post-rut (sample), and post-rut (negative control) urine. During a 10-minute non-replicated trial, female moose spent significantly ($F = 16.35$, d.f. = 2, 9, $P = 0.001$) more time engaging in Neutral behaviors (i.e., walking, standing) than investigating the sample or the control. The mean time spent engaging in Neutral behaviors was 177 seconds (SD±71), which comprised 83% of the trial. In comparison, female moose spent on average 27 seconds (SD±33), which comprised 13% of the trial, and 8.5 (SD±7) seconds which comprised 4% of the trial, investigating the sample, and control, respectively (Figure 3.7).

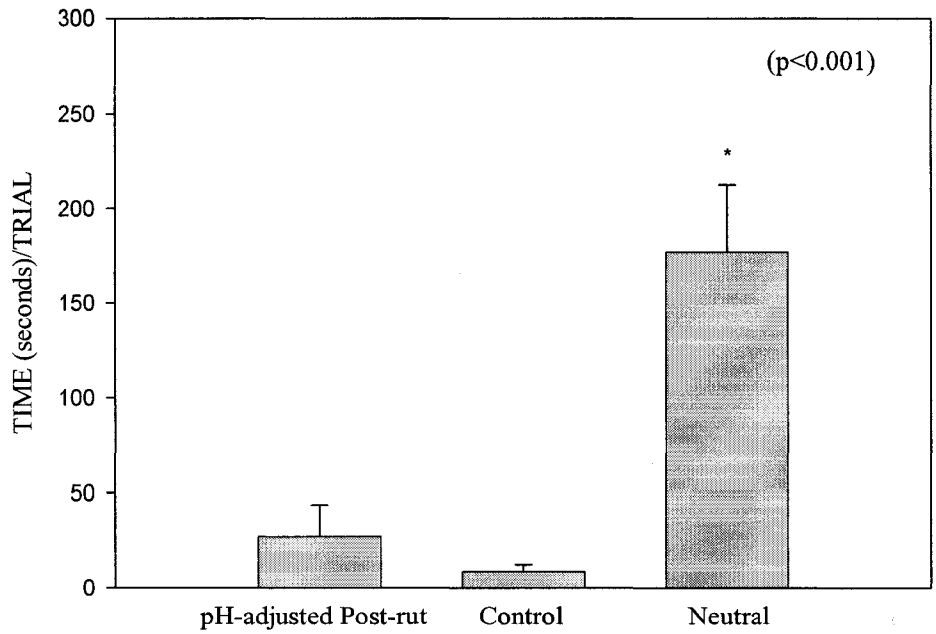

Figure 3.7 Experiment 1: Female response to pH-adjusted post-rut urine and control (post-rut urine) (n=4). Mean time spent (±SE) investigating pH-adjusted post-rut, control samples, and engaging in Neutral behaviors during a 10-minute trial.

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Experiment 2 tested the response of females to pH-adjusted post-rut (sample), and rut (positive control) urine. During a 10-minute non-replicated trial, female moose spent significantly more time investigating the control ($F = 6.45$, d.f. = 2, 9, $P = 0.01$) than the sample, or engaging in Neutral behaviors. In comparison, female moose spent on average 32 (SD±26) seconds, which comprised 8% of the trial, and 142 (SD±41) seconds engaging in Neutral behaviors, which comprised 34% of the trial, respectively (Figure 3.8).

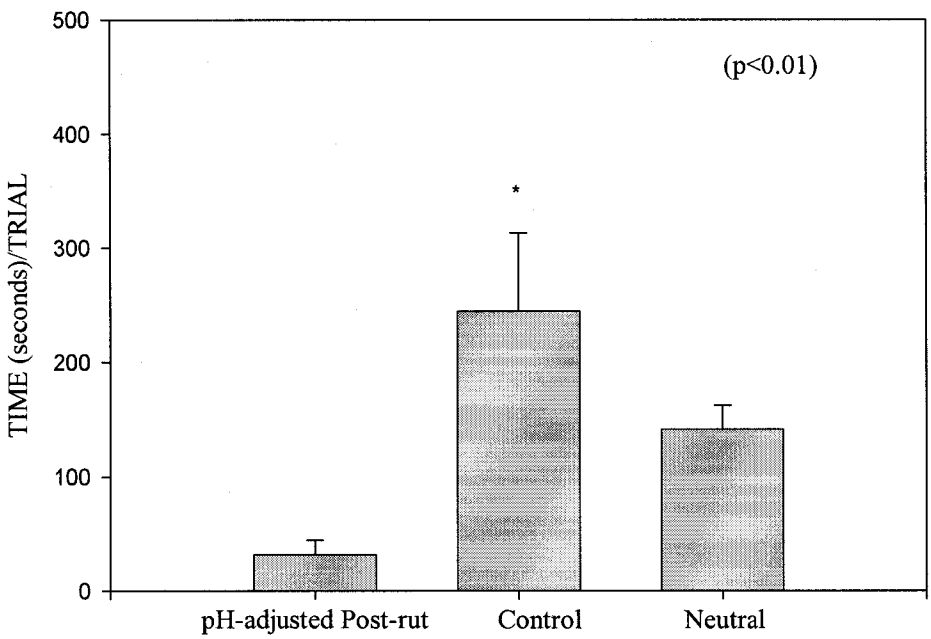

Figure 3.8 Experiment 2: Female response to pH-adjusted post-rut urine and positive control (rut urine) (n=4). Mean time spent (±SE) investigating pH-adjusted post-rut, control samples, and engaging in Neutral behaviors during a 10-minute trial.

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Experiment 3 tested the response of females to *p*-cresol and geraniol in pH-adjusted post-rut (Cocktail), and post-rut (negative control) urine. During a 10-minute non-replicated trial, female moose did not spend significantly ($F = 2.16$, d.f. = 2, 9, $P = 0.17$) more time investigating the sample, than the control or engaging in Neutral behaviors. Female moose spent on average 11, and 85 seconds investigating the sample, and control, respectively. The mean time spent engaging in Neutral behaviors was 224 seconds (Figure 3.9).

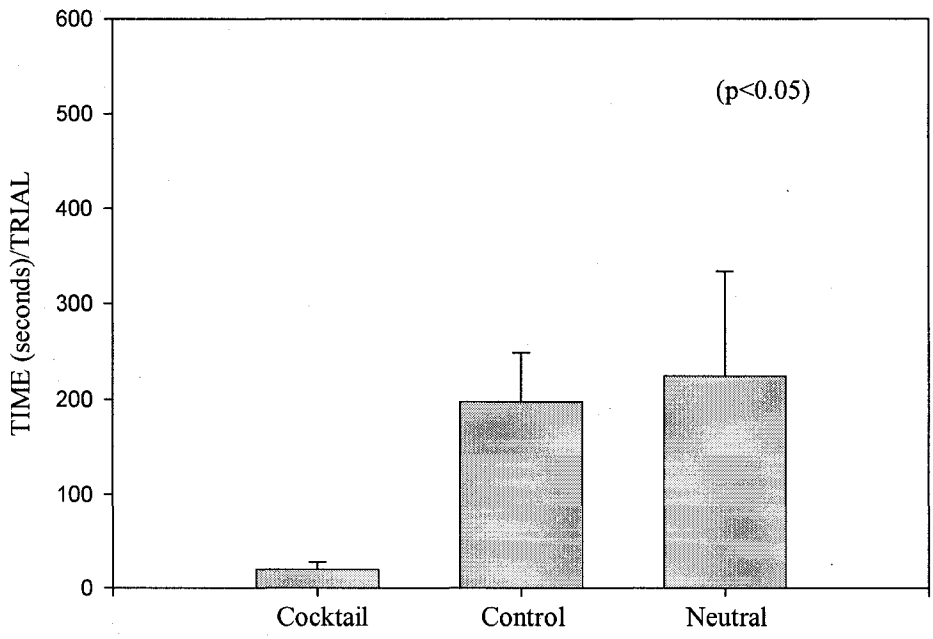

Figure 3.9 Experiment 3: Female response to Cocktail and positive control (rut urine) (n=4). Mean time spent (±SE) investigating Cocktail, control, and engaging in Neutral behaviors during a 10-minute trial.

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Experiment 4 tested the response of females to the Cocktail and rut urine (positive control). During 10-minute replicated trials, female moose spent significantly ($F = 11.85$, d.f. = 1, 22, $P = 0.002$) more time investigating the control than the Cocktail. The mean time females spent investigating the Cocktail was 19 seconds (SD±27), which comprised 9% of the trial. In contrast, females on average spent 197 (SD±177) seconds investigating the control, which comprised 81% of the trial (Figure 3.10).

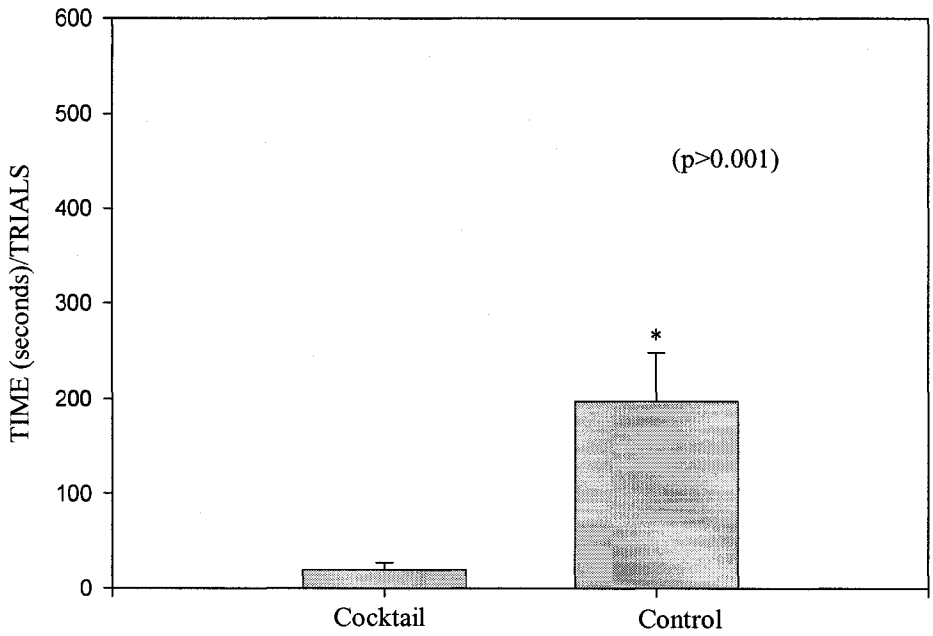

Figure 3.10 Experiment 4: Female response to Cocktail and positive control (rut urine) (n=4). Mean time spent (±SE) investigating Cocktail and control during 10-minute replicated trials.

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3.5 Test of Bioactivity of 3 Unique Preparatory Gas Chromatography Effluents

Experiment 1: Whole fraction

During a 10-minute non-replicated trial for seven female moose, females spent significantly ($F = 26.90$, d.f. = 1, 12, $P = 0.0002$) more time investigating the Whole fraction than the control (Figure 3.11). Mean time spent investigating the sample was 255 seconds (SD±105), which comprised 84% of the trial. In contrast, the mean time spent investigating the control was 47 seconds (SD±23.3), which comprised 16% of the trial.

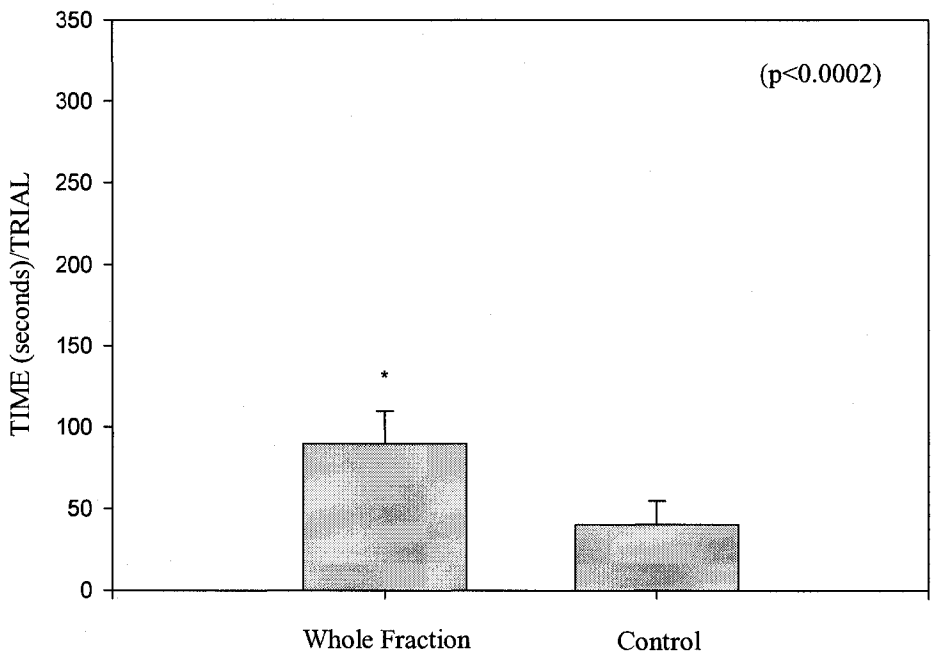

Figure 3.11 Experiment 1: Female response to preparatory gas chromatography Whole fraction and control (n=7). Mean time spent (±SE) investigating Cocktail, control, and engaging in Neutral behaviors during a 10-minute trial.

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The sample elicited characteristic behaviors (e.g., wallowing) when females are exposed to pure rut urine. Additionally, this fraction contained more volatile compounds than Fractions 1, 2, and 3 (Figures 3.12 and 3.13).

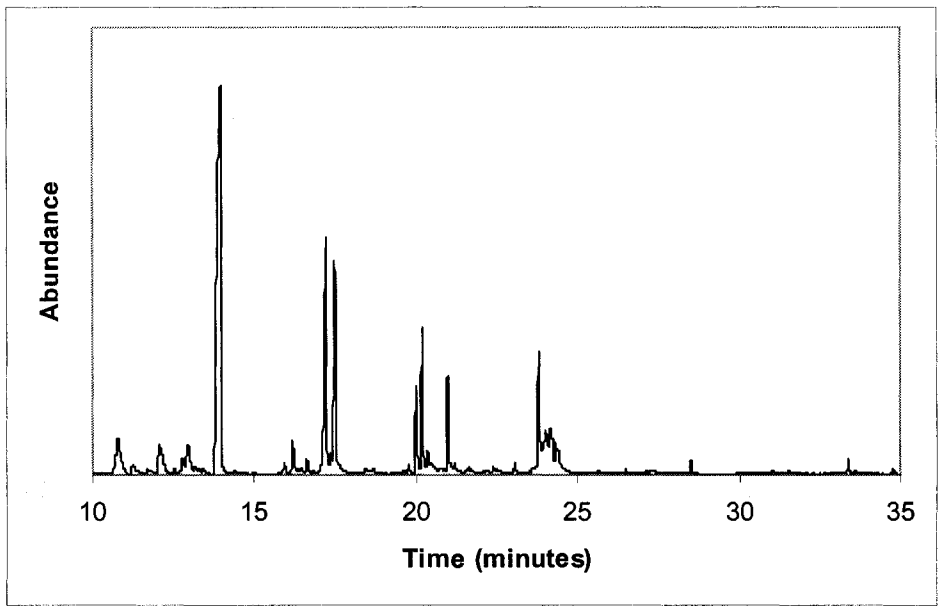

Figure 3.12 Chromatograph of the effluent collected by preparatory gas chromatography over the entire 30-minute period, termed the: 'Whole' fraction.

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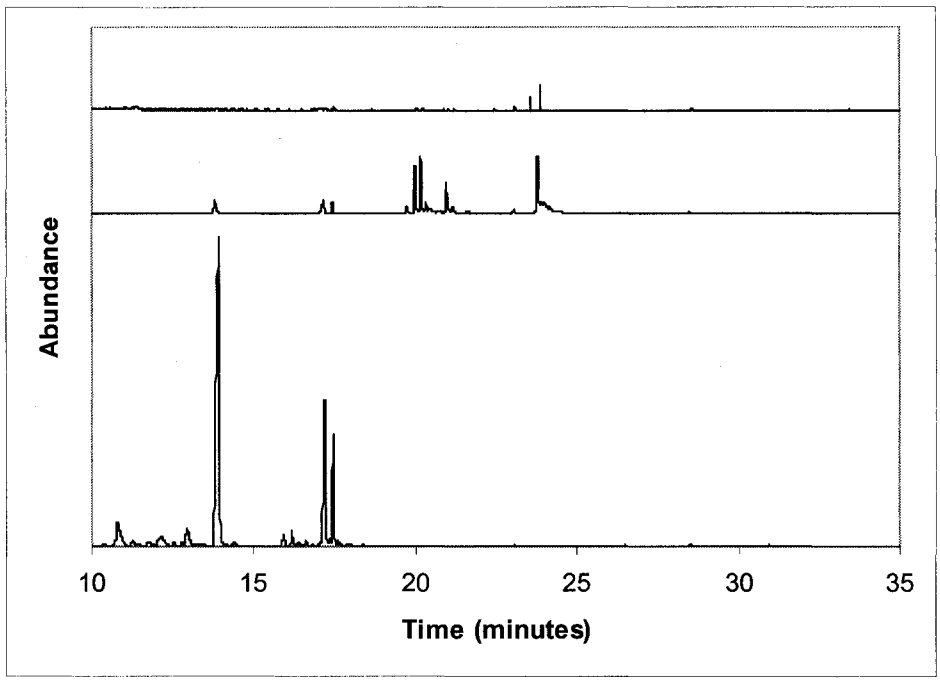

Figure 3.13 Chromatograph of the effluent collected by preparatory gas chromatography. The upper trace represents the effluent collected over the third 10 minutes, termed: 'Fraction 3'. The trace was enlarged 10 times to scale with the other 2 traces; the middle trace represents the effluent collected over the first 10 minutes, termed: 'Fraction 1'; and the lower trace represents the effluent collected over the second 10 minutes, termed: 'Fraction 2'.

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Experiment 2: Fraction 1

During 10-minute replicated trials for seven female moose, females spent significantly ($F = 4.53$, d.f. = 1, 40, $P = 0.0007$) more time investigating Fraction 1 than the control. Mean time spent investigating the sample was 90 seconds (SD±91), which comprised 69% of the trials. In contrast, the mean time spent investigating the control was 41 seconds (SD±66), which comprised 31% of the trials (Figure 3.14). This Fraction was less complex than the Whole. Moreover, the sample also elicited characteristic behaviors (e.g., wallowing) when females are exposed to rut urine.

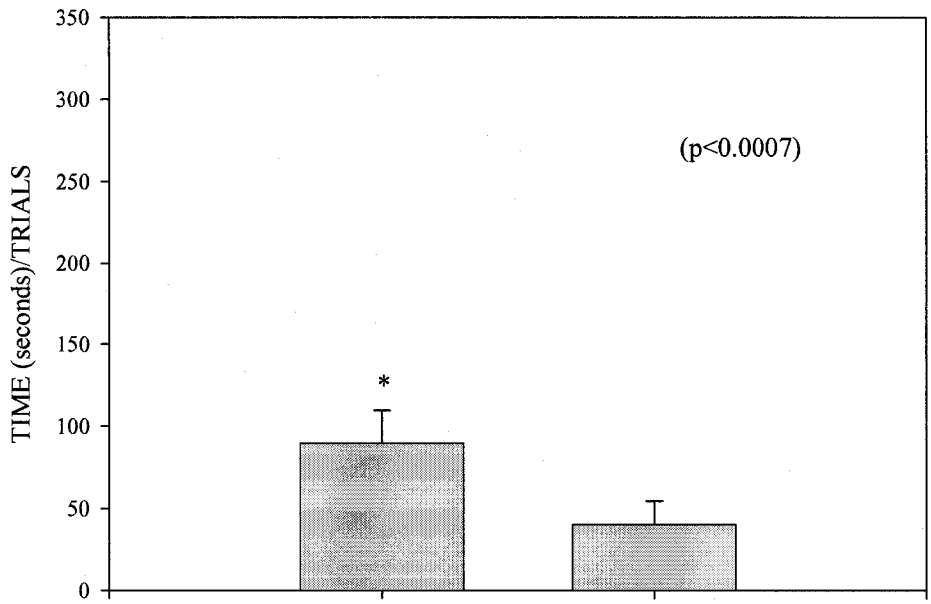

Figure 3.14 Experiment 2: Female response to preparatory gas chromatography Fraction 1 and control (n=7). Mean time spent (±SE) investigating Fraction 1 and control during 10-minute trials.

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Experiment 3: Fraction 2

During 10-minute replicated trials for seven female moose, females spent significantly ($F = 13.87$, d.f. = 1, 40, $P = 0.0006$) more time investigating Fraction 2 than the control. Mean time spent investigating the sample was 94 seconds (SD±85), which comprised 81% of the trials. In contrast, the mean time spent investigating the control was 22 seconds (SD±21), which comprised 19% of the trials (Figure 3.15). Fraction 2 contained more volatiles than Fraction 1. The sample also elicited the less also elicited the characteristic behaviors (e.g., wallowing) when females are exposed to rut urine.

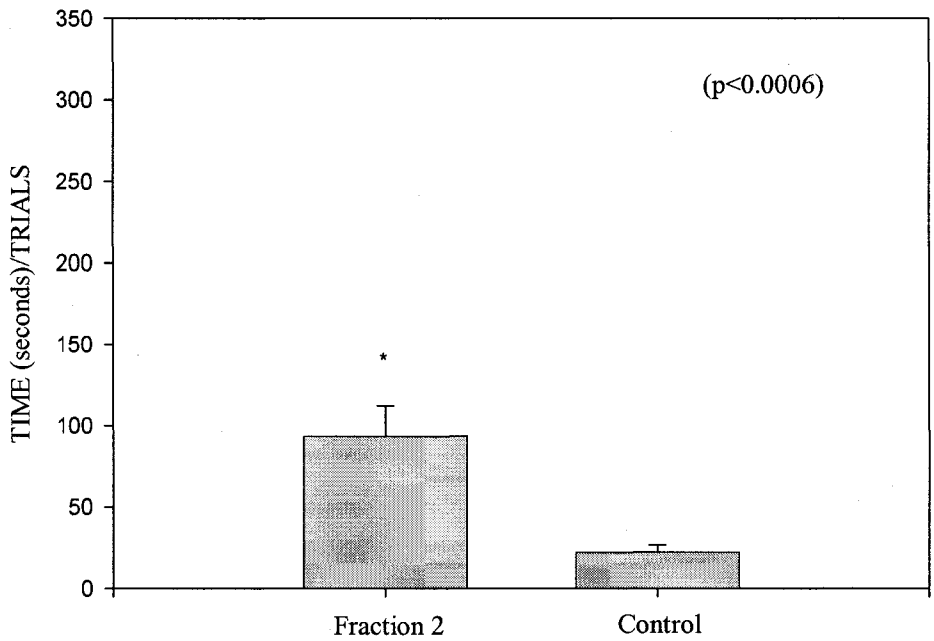

Figure 3.15 Experiment 3: Female response to preparatory gas chromatography Fraction 2 and control (n=7). Mean time spent (±SE) investigating Fraction 2 and control during 10-minute trials.

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Experiment 4: Fraction 3

During 10-minute replicated trials for seven female moose, females spent significantly ($F = 7.65$, d.f. = 2, 60, $P = 0.0001$) more time engaging in Neutral behaviors than either investigating the sample, or the control (Figure 3.16). Mean time spent investigating the sample was 92 seconds (SD±98), which comprised 27% of the trials, and investigating the control was 46 seconds (SD±71), which comprised 13% of the trials. In contrast, the mean time spent engaging in Neutral behaviors was 206 seconds (SD±155), or 60% of the trials. The sample contained fewer volatiles than either Fraction 1 or 2. Unlike Experiments 1, 2, and 3, This Fraction did not elicit the characteristic behaviors (e.g., wallowing) observed when female moose are exposed to rut urine.

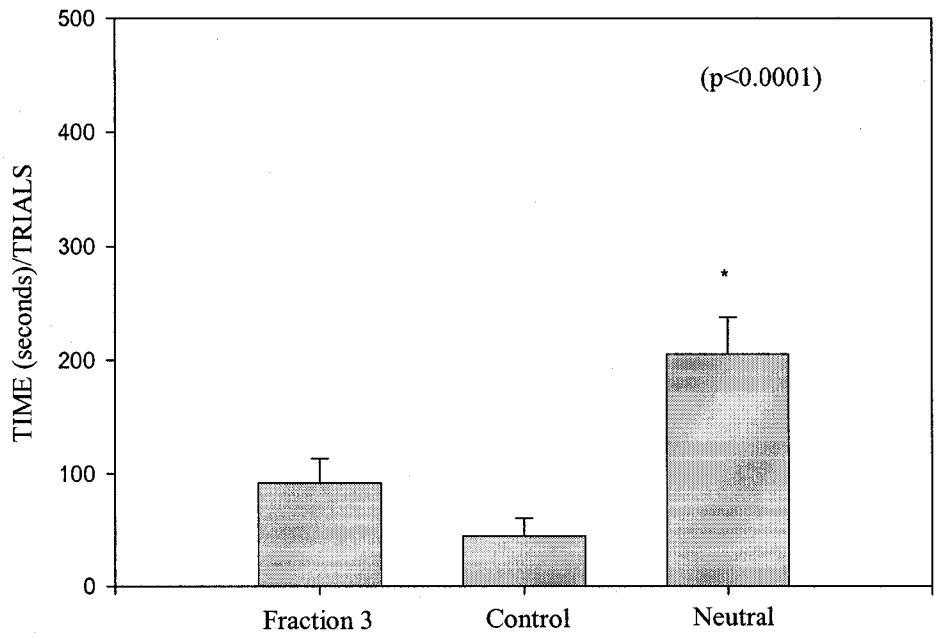

Figure 3.16 Experiment 4: Female response to preparatory gas chromatography Fraction 3, control, and engaging in Neutral behaviors (n=7). Mean time spent (±SE) investigating Fraction 3, control, and engaging in Neutral behaviors during 10-minute trials.

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3.6 Test of Bioactivity of 3α-Hydroxy-5β-androstan-17-one (steroid), and Flash Chromatography Eluants

Experiment 1: Steroid (concentration 0.25 mg/ml)

During a 10-minute non-replicated trial for three female moose, females spent significantly ($F = 9.73$, d.f. = 2, 6, $P = 0.01$) more time engaging in Neutral behaviors than investigating the control, and the sample. Mean time spent engaging in Neutral behaviors was 297 seconds (SD±155), which comprised 93% of the trials. In comparison, the mean time spent investigating the sample was 4 seconds (SD±6), which comprised 1% of the trials, and the mean time spent investigating the control was 20 seconds (SD±29), which comprised 6% of the trials (Figure 3.17).

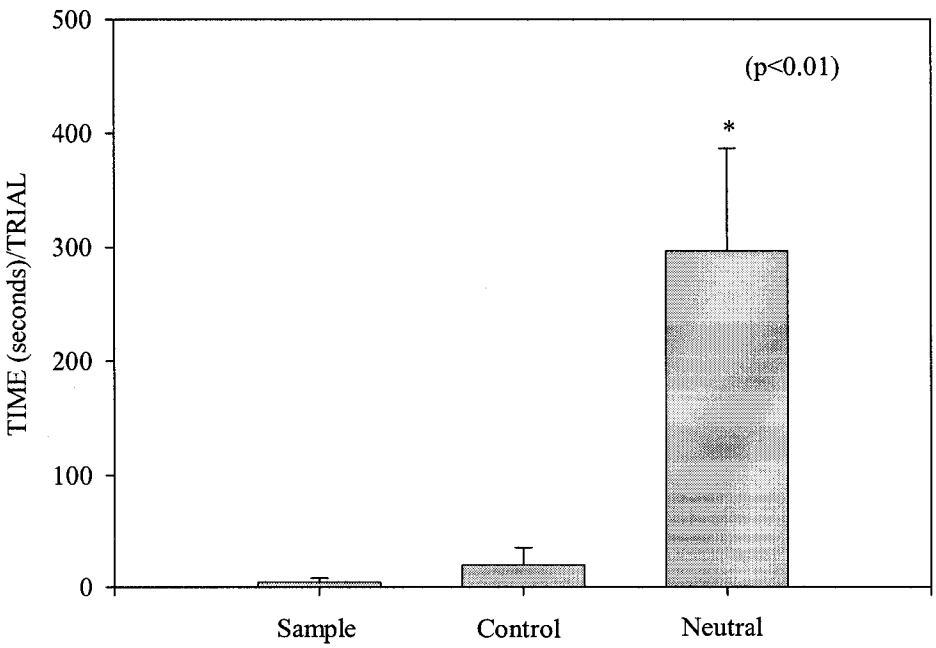

Figure 3.17 Experiment 1: Female response to the Steroid (concentration 0.25 mg/ml) and control (n=3). Mean time spent (±SE) investigating the Steroid and control during a 10-minute trial.

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Experiment 1a: Steroid (concentration 18 mg/ml)

During 10-minute replicated trials for three female moose, females spent significantly ($F = 11.10$, d.f. = 2, 24, $P = 0.005$) more time engaging in Neutral behaviors than investigating the control, and the sample. Mean time spent engaging in Neutral behaviors was 232 seconds (SD±199), which comprised 92% of the trials. In comparison, the mean time spent investigating the sample was 15 seconds (SD±28), which comprised 6% of the trials, and the mean time spent investigating the control was 4 seconds (SD±3), which comprised 2% of the trials (Figure 3.18).

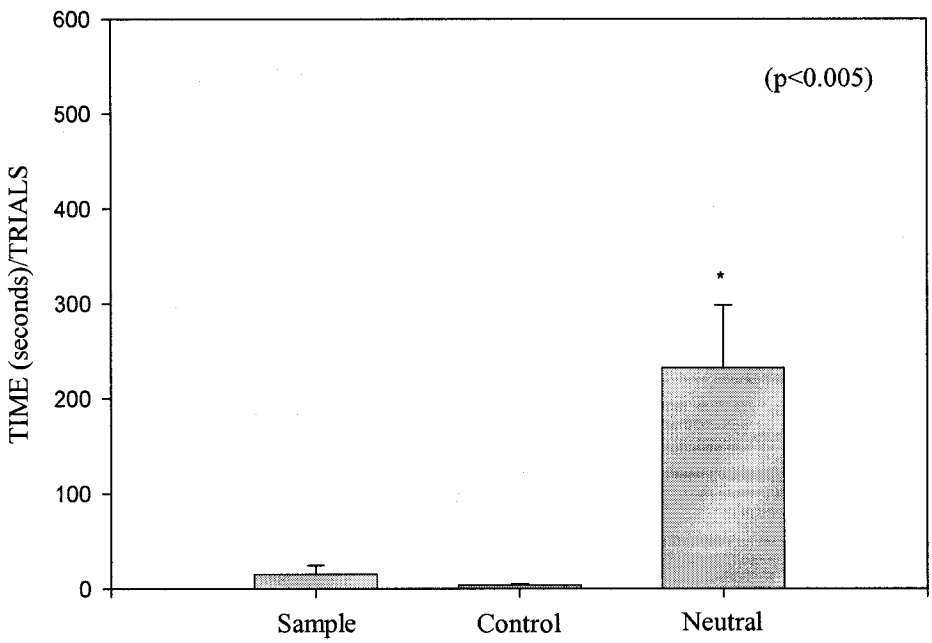

Figure 3.18 Experiment 1a: Female response to the Steroid (concentration 18 mg/ml) and control (n=3). Mean time spent (±SE) investigating the Steroid and control during 10-minute trials.

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Experiment 2: Steroid (concentration 18 mg/ml) and *p*-cresol (Cocktail)

During a 10-minute unreplicated trial for three female moose, females spent significantly ($F = 8.83$, d.f. = 2, 6, $P = 0.01$) more time engaging in Neutral behaviors than investigating the control, and the Cocktail. Mean time moose spent engaged in Neutral behaviors was 200 seconds (SD±113), which comprised 94.4% of the trials. In comparison, the mean time spent investigating the Cocktail was 8.3 seconds (SD±6), which comprised 3.8% of the trials, and the mean time investigating the control was 4 seconds (SD±5), which comprised 1.9% of the trials (Figure 3.19).

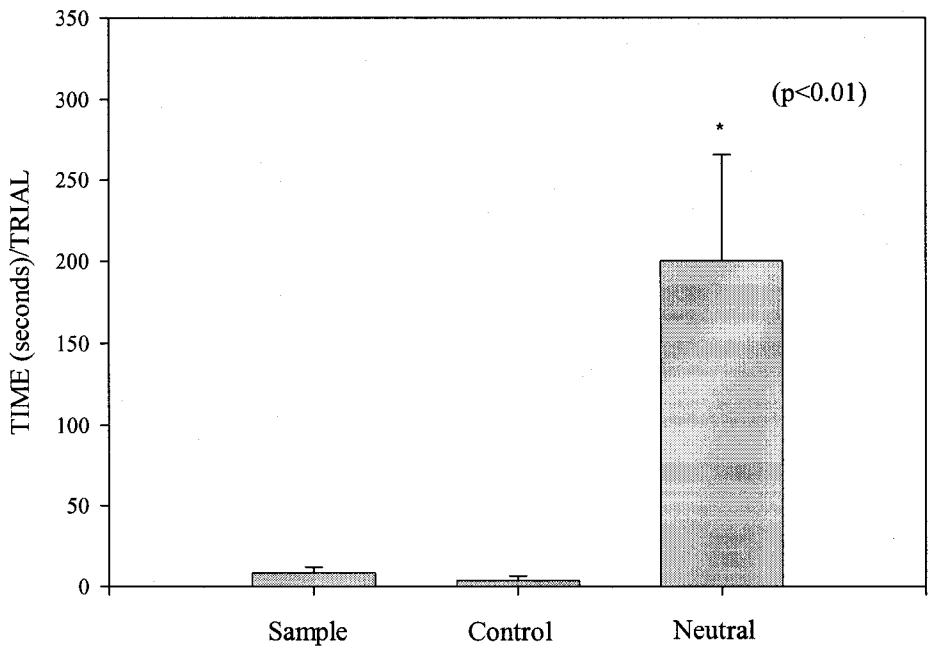

Figure 3.19 Experiment 2: Female response to the Cocktail and control (n=3). Mean time spent (±SE) investigating the Cocktail and control during a 10-minute trial.

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Experiment 3: Steroid (concentration 18 mg/ml) and positive control

During a 10-minute unreplicated trial for three female moose, females spent significantly ($F = 3.9$, d.f. = 2, 12, $P = 0.05$) more time engaging in Neutral behaviors than investigating the control, and the sample. Mean time spent engaging in Neutral behaviors was 234 seconds (SD±110), which comprised 46.3% of the trials. In comparison, the mean time investigating the sample was 108 seconds (SD±86), which comprised 21.4% of the trials, and the mean time investigating the control was 163 seconds (SD±40), which comprised 32.3% of the trials (Figure 3.20).

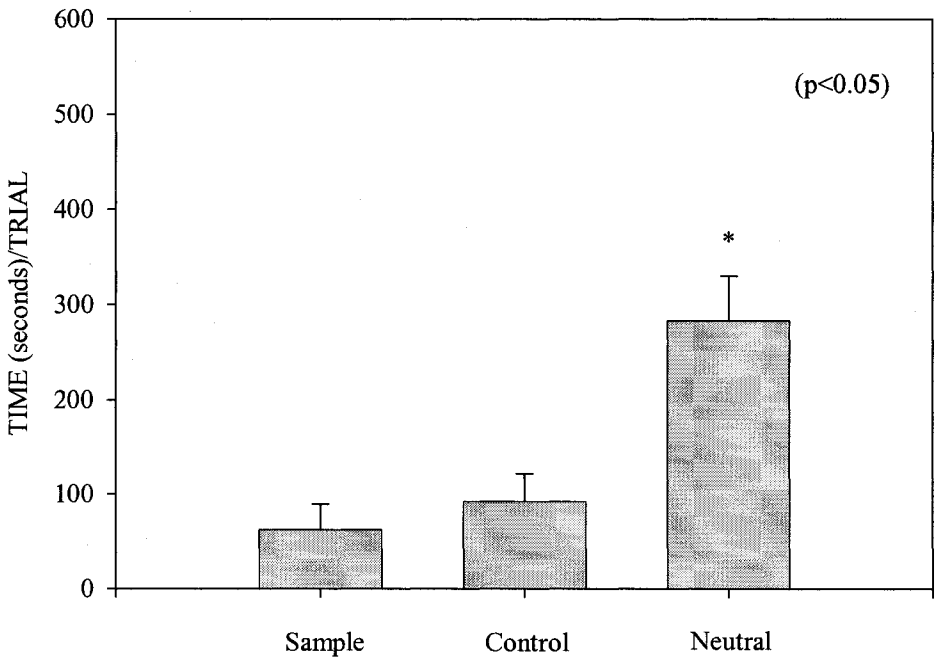

Figure 3.20 Experiment 3: Female response to the Steroid (concentration 18 mg/ml) and control (n=3). Mean time spent (±SE) investigating the Steroid and positive control during a 10-minute trial.

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Experiment 4: Steroid (concentration 18 mg/ml) in methanol

During 10-minute replicated trials for three female moose, females spent significantly ($F = 31.40$, d.f. = 2, 24, $P = 0.0001$) more time engaging in Neutral behaviors than investigating the control, and the sample. Mean time spent engaging in Neutral behaviors was 326 seconds (SD±157), which comprised 90% of the trials. In comparison, the mean time spent investigating the sample was 33 seconds (SD±53), which comprised 9.1% of the trials, and the mean time spent investigating the control was 3 seconds (SD±6), which comprised 0.83% of the trials (Figure 3.21).

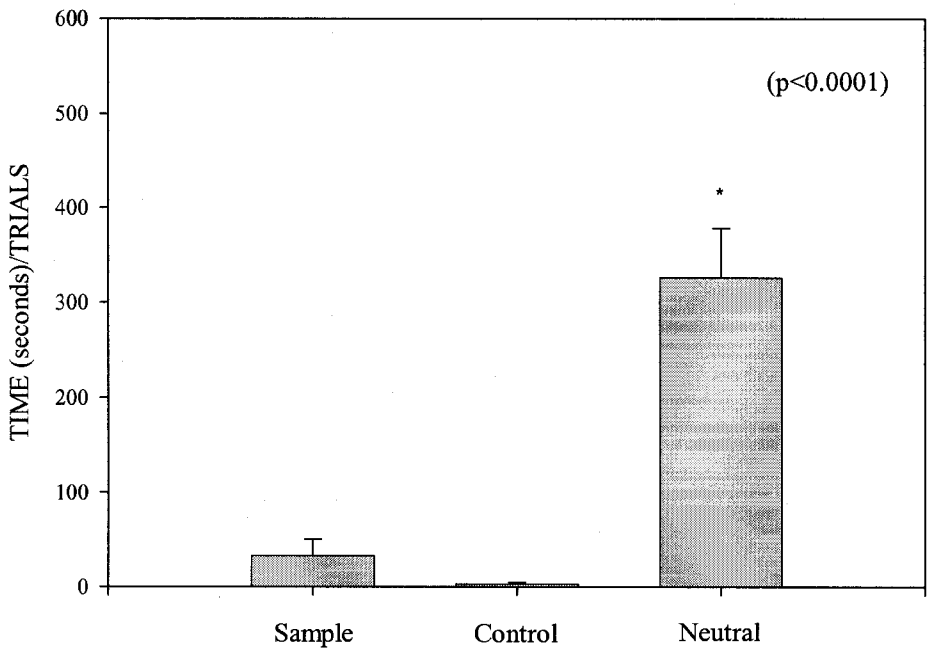

Figure 3.21 Experiment 4: Female response to the Steroid (concentration 18 mg/ml) and control (n=3). Mean time spent (±SE) investigating the Steroid and control during 10-minute trials.

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Experiment 5: Flash Chromatography Fraction A13-17

During five 10-minute trials for five female moose, females spent significantly ($F = 18.16$, d.f. = 1, 28, $P = 0.0002$) more time investigating the sample than the control. Mean time spent investigating the sample was 225 seconds (SD±171), which comprised 87.2% of the trials. In contrast, the mean time that females spent investigating the control was 33 seconds (SD±30), which comprised 12.8% of the trials (Figure 3.22).

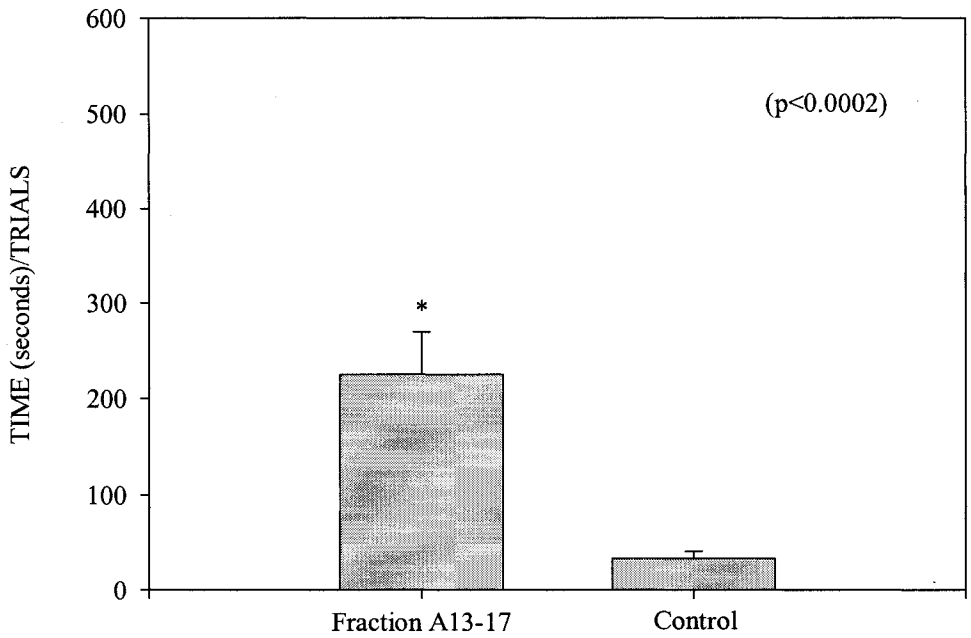

Figure 3.22 Experiment 5: Female response to flash chromatography Fraction A13-17 (n=5). Mean time spent (±SE) investigating Fraction A13-17, and control during 10-minute replicated trials. Description of Fraction A13-17 in Chapter 2: General Experimental Procedures.

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The sample elicited characteristic behaviors (e.g., wallowing) when females are exposed to pure rut urine. Additionally, this fraction contained more volatile compounds than Fractions B3-11, and B12-28 and C1-7 (Figure 3.23).

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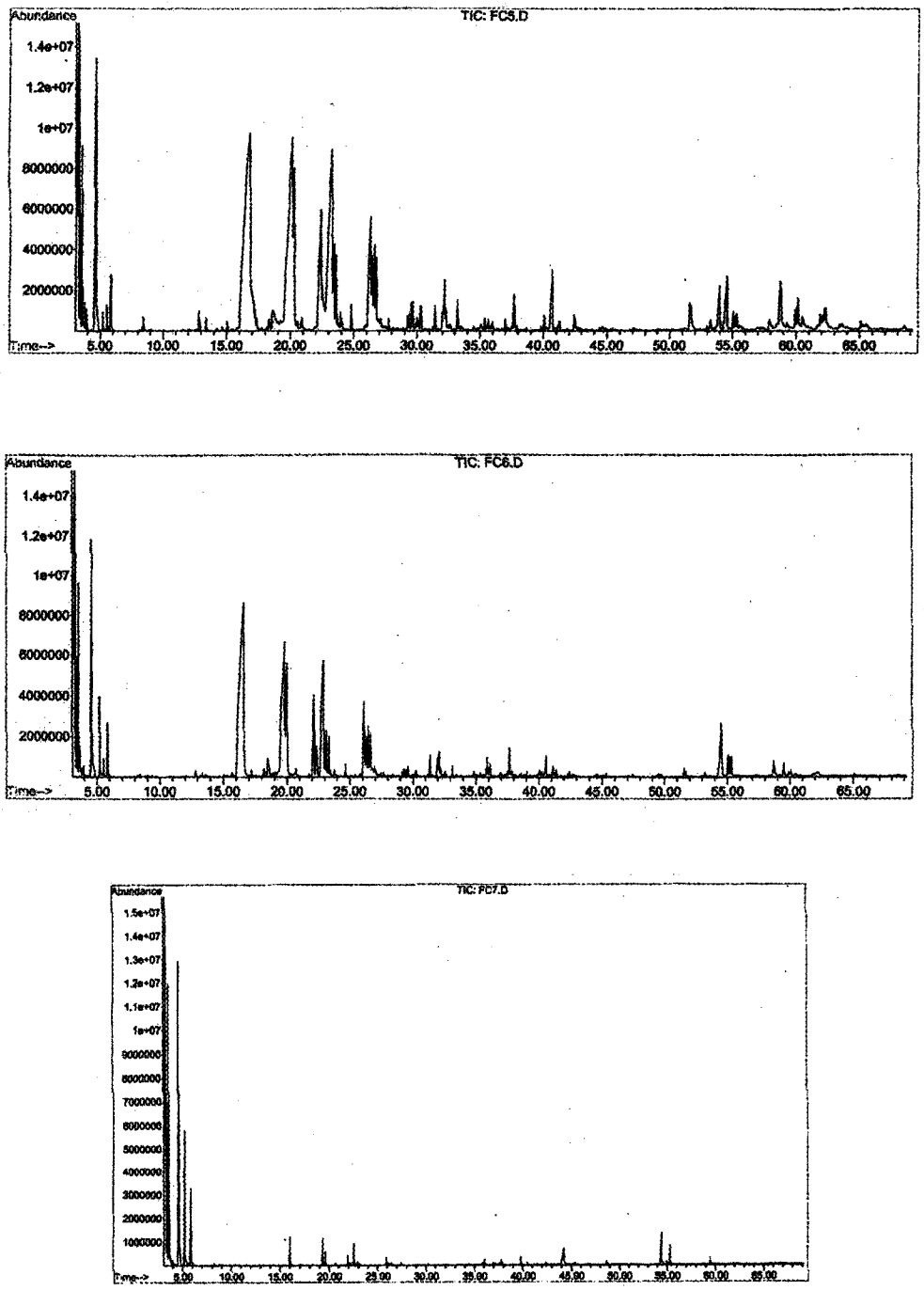

Figure 3.23 Chromatographs of flash chromatography Experiments 5, 6, and 7. Upper trace represents Experiment 5, Fractions A13-17 and B1-2; middle trace represents Experiment 6, Fraction B3-11; bottom trace represents Experiment 7, Fractions B12-18 and C1-C7.

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Experiment 6: Flash Chromatography Fraction B3-11

During five 10-minute trials for five female moose, females spent significantly ($F = 14.52$, d.f. = 2, 42, $P = 0.0001$) more time engaging in Neutral behaviors than investigating the control, and the sample ($F = 9.31$, d.f. = 1, 28, $P = 0.004$). Mean time spent engaging in Neutral behaviors was 287 seconds (SD±154), which comprised 65% of the trials. In comparison, the mean time that females spent investigating the control was 40 seconds, which comprised 9% of the trials; and the mean time that females spent investigating the sample was 115 seconds (SD±154), which comprised 26% of the trials (Figure 3.24).

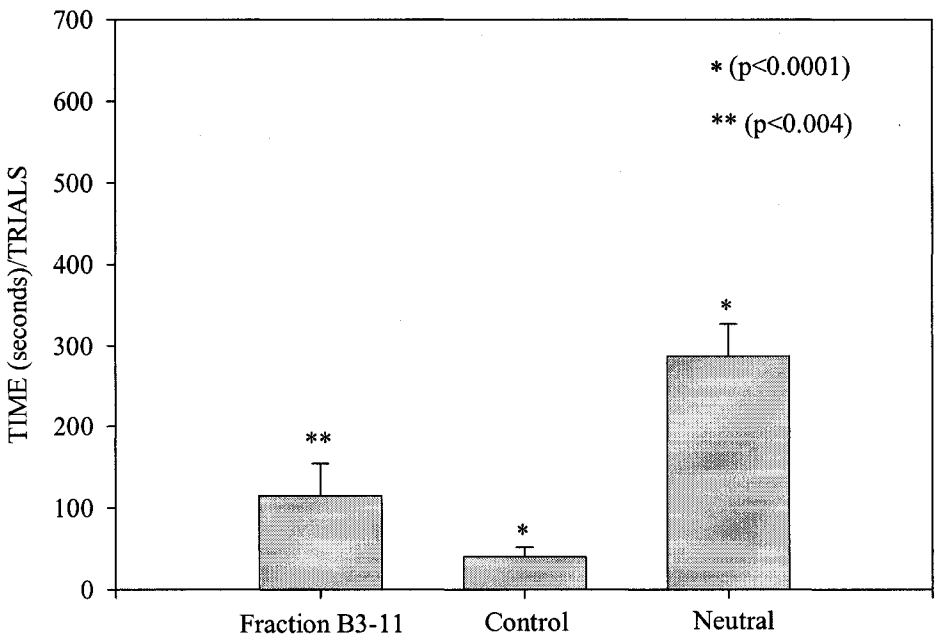

Figure 3.24 Experiment 6: Female response to flash chromatography Fraction B3-11 (n=5). Mean time spent (±SE) investigating Fraction B3-11, control, and engaging in Neutral behaviors during 10-minute replicated trials. Description of Fraction B3-11 in Chapter 2: General Experimental Procedures.

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Experiment 7: Flash Chromatography Fractions B12-18 and C1-7

During five 10-minute trials for five female moose, females spent significantly ($F = 72.95$, d.f. = 2, 42, $P = 0.0001$) more time engaging in Neutral behaviors than investigating the control, and the sample. Mean time spent engaging in Neutral behaviors was 374 seconds (SD±157), which comprised 89% of the trials. In comparison, the mean time spent investigating the sample was 27 seconds (SD±38), which comprised 6.4% of the trials, and the mean time spent investigating the control was 19 seconds (SD±36) that comprised 4.5% of the trials (Figure 3.25).

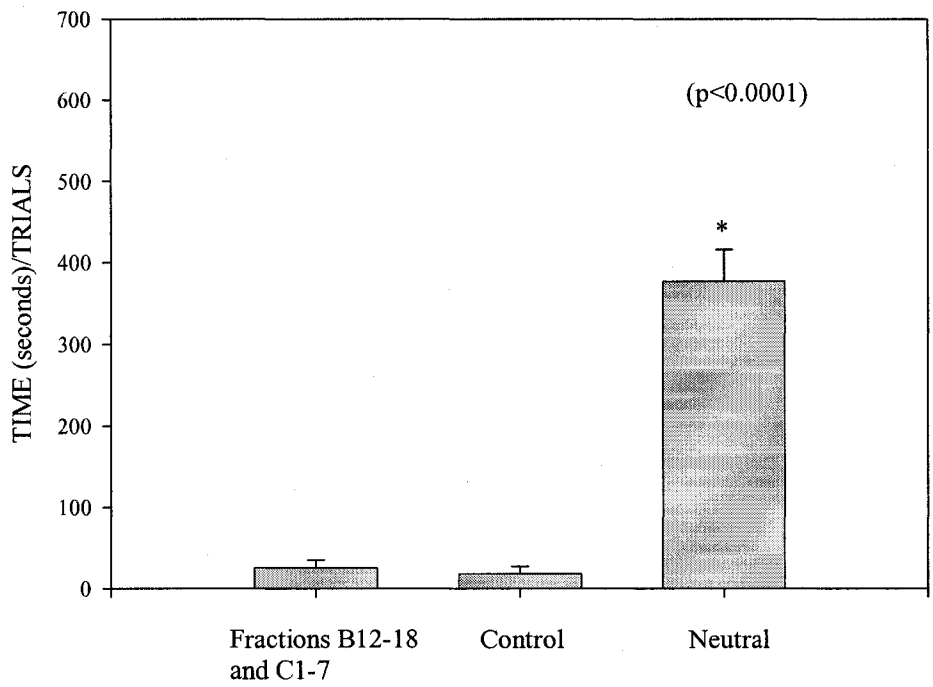

Figure 3.25 Experiment 7: Female response to flash chromatography Fractions B12-18 and C1-7 (n=5). Mean time spent (±SE) investigating Fractions B12-18 and C1-7, control, and engaging in Neutral behaviors during 10-minute replicated trials. Description of Fractions B12-18 and C1-7 in Chapter 2: General Experimental Procedures.

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CHAPTER 4

DISCUSSION

4.1 Test of Bioactivity of Pre-rut Versus Rut Urine

Urine is an important chemical cue used by male moose during the rut (Miquelle and Van Ballenberghe, 1985; Miquelle, 1991; Van Ballenberghe and Miquelle, 1993). No studies, however, have established that female moose prefer the urine from rut urine to non-rut urine. This study experimentally demonstrated that age, or dominance is not a factor because the urine was obtained from the same males during the pre-rut, and rut periods.

I hypothesized that when female moose were presented with urine from the pre-rut, and rut periods, females would spend significantly more time investigating rut urine.

Female moose spent significantly more time investigating the rut sample than the pre-rut sample (Figure 3.1).

This experiment documented that females responded markedly to constituent(s) in adult male moose urine rut by wallowing; however, one hallmark behavior (e.g., wallowing) was not observed when female moose investigated the pre-rut sample. This is the first experimental evidence that urine from rut elicits wallowing behaviors in females independent of age, and male dominance, which was held constant by the experimental design. The results from this study strongly infer that pheromone(s) were present in the samples presented to female moose.

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4.2 Solid Phase Microextraction (SPME) Headspace Analysis

To understand the principles involved in this experiment, an overview on SPME headspace analysis theory is appropriate, and is provided below.

Analyses of volatile organic compounds by SPME headspace analysis has been used in a wide-range of applications (Scheppers Wercinski, 1999). Headspace analysis by SPME may also be used as an extraction technique for organic compounds in aqueous samples such as moose urine. Volatile analytes are adsorbed directly from the headspace above the sample onto a fiber. Once the fiber is exposed to the headspace, the analytes partition from the sample matrix into the stationary phase until equilibrium is reached between the fiber coating and the volatiles in the headspace above the aqueous phase. The fiber coating determines the adsorption of analytes, and the rate of adsorption is directly related to the volatility of analytes in the aqueous sample. Typically, more volatile samples adsorb on the fiber faster, but less volatile substances are more efficiently captured if equilibrium is allowed to be established (Scheppers Wercinski, 1999).

Some disadvantages of SPME headspace analysis include: 1) knowledge of the chemical profile (e.g., molecular weights between 40-300 Daltons) of the sample to be examined to use the appropriate fiber type for the analysis; 2) sensitivity is dependent on the fiber's ability to adsorb analytes that are related to vapor pressure, functional group, concentration, polarity, and detector type (Scheppers Wercinski, 1999); 3) extraction efficiency may be highly variable and compound dependent. Nonetheless, if the GC column and fiber have the same phase, the relative extraction efficiencies can be easily

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calculated based on retention times (Scheppers Wercinski, 1999); and 4) automatic samplers are available; however, they are very expensive.

Despite some of the disadvantages associated with SPME headspace analysis, there are several advantages. Compared with the conventional methods of solvent extraction, collection and analysis of volatiles by SPME does not yield a large solvent peak in the gas chromatographic analysis that may obstruct the detection of highly volatile substances that can co-elute with the solvent. Therefore, data collected from SPME-related headspace analyses can serve as an additional analytical method to augment solvent-based extractions. Other advantages associated with SPME headspace analysis include: 1) no artifacts associated with solvent impurities (Pawliszyn, 1997); 2) no harsh treatment of the sample needed for solvent concentration (e.g., rotoevaporation with heat) which may also result in concomitant loss of other substances; 3) minimal labor involved in sample preparation; 4) small amount of sample is required for analysis; and 5) disposal of sample is environmentally friendly.

The urine from adult male moose has a suite of volatile compounds (Whittle, 1999). I wanted to characterize the urinary profiles of urine from three periods (pre-rut, rut, and post-rut) before performing solvent-based extractions. I hypothesized that the urine from adult male moose during rut would contain more volatile compounds in elevated levels than from males during the pre-rut and post-rut periods.

Data from these experiments support my previous research (Whittle et al., 2000). I observed the same elevated levels of volatile compounds associated with rut urine in contrast to pre-rut. Furthermore, several compounds, i.e., *p*-cresol and dimethyl sulfone,

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were present in my previous study as well as in this analysis. Nonetheless, I was unable to detect some compounds reported by Whittle et al. (2000) that were subsequently characterized as artifacts. Therefore, SPME headspace analysis is an excellent analytical tool for adjunct studies carried out in conjunction with solvent-based extractions.

4.3 Test of Bioactivity of Pentane Extracted Rut Urine

There are qualitative and quantitative differences between urine of adult male moose during the rut and nonrut periods (Whittle et al., 2000). Additionally, urine from the rut is a complex mixture of well over 100 compounds (Whittle, 1999). To determine which urinary constituents from rut urine may serve as the putative pheromone, it was essential that urine would retain bioactivity after chemical techniques. This was a necessary step to facilitate partitioning of the bioactive components that would be presented to female moose during subsequent behavioral bioassays. I hypothesized that when presented with pentane, dichloromethane, and ethyl acetate extracts from rut urine, female moose would respond to one of the three extracts.

GC/MS analyses of the 3 extracts indicated that the pentane extract contained more volatile compounds and these were in elevated levels when compared to the dichloromethane, or ethyl acetate extracts (Figure 3.5). Although there was no significant difference between the mean times female moose spent investigating the 3 extracts, the pentane extract significantly elicited wallowing behaviors of females (Figure 3.6). The more complex composition, and elevated levels of compounds in the pentane extract may explain this behavior.

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The results from this study support the hypothesis that rut urine can be chemically extracted and maintain its bioactivity when presented to female moose. Furthermore, these results provide information on the chemical and physical nature of the chemosignal. The bioactive urinary constituent(s) can be extracted with organic solvents such as pentane, are not a protein, or carbohydrate, are relatively non-polar, and have low molecular weights. Typically, airborne pheromones usually contain 5-20 carbon atoms, must be volatile to reach the receiver, and have molecular weights near 300 (Bradbury and Vehrencamp, 1998). Compounds found in the pentane extract meet the criteria for pheromones, and suggests that the putative pheromone was present in this extract.

4.4 Test of Bioactivity of *p*-Cresol, and Geraniol (Cocktail)

Two major constituents (*p*-cresol, and geraniol) present in rut urine were identified by SPME headspace analyses. Although, *p*-cresol and geraniol both occur in nonrut urine, they are not present at the elevated levels in rut urine. Para-cresol has been identified as a component of the pheromone in two species of hard tick (*Rhipicephalus appendicalatus* and *R. pulchellus*) (Wood et al., 1975) and cabbage looper (*Trichoplusia ni*) (Heath et al., 1992). Insect pheromones also may function as pheromones in mammals; Rasmussen et al. (1997) demonstrated that a lepidopteran-like pheromone occurred in the urine of female Asian elephants, and that compound was identified as a female-to-male pheromone. Perhaps, a similar mechanism of convergent evolution is involved in the elevated levels of *p*-cresol that occurs in rut urine, and *p*-cresol has pheromonal activity. Because both *p*-cresol and geraniol were present at elevated levels in rut urine, they seemed likely candidates for the putative pheromone.

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I hypothesized that when presented with these two compounds female moose would respond. To test this hypothesis, however, it was important that *p*-cresol and geraniol (Cocktail) be presented in urine that did not have bioactivity (e.g., nonrut urine). Furthermore, the pH of the nonrut urine was adjusted to that of rut urine. Adjustment of nonrut to the pH of rut urine was conducted to ensure substances in nonrut urine were in the same ionization state as found in rut urine.

Two major urinary constituents (*p*-cresol, and geraniol) of rut urine identified by SPME headspace analyses did not elicit a significant response when presented to female moose. Female moose spent more time investigating the control than the Cocktail. As determined by SPME headspace analysis, *p*-cresol and geraniol occur in high abundance in rut urine. Although both compounds occur in nonrut urine, they are present at a much lower concentrations.

Para-cresol is an end product of anaerobic microbial degradation of tyrosine, which occurs in the rumen (Bone et al., 1976; Martin, 1982). During rut, adult male moose become hypophagic and rely upon the catabolism of endogenous reserves to meet their metabolic needs (Miquelle, 1990). This hypophagic behavior may explain the quantitative differences of *p*-cresol observed between rutting and nonrutting male urine (Whittle, et al. 2000).

The combination of geraniol and *p*-cresol failed to elicit a significant response from female moose. This outcome, however, does not preclude their use as a chemical signal. Perhaps, geraniol and *p*-cresol alone or together may act synergistically in

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combination with other compound(s) in rut urine. At the very least, geraniol and *p*-cresol, which are odoriferous, may add to the unique bouquet that constitutes rut urine.

4.5 Test of Bioactivity of 3 Unique Preparatory Gas Chromatography Effluents

Results from a previous behavioral bioassay (Section 3.3) demonstrated that female moose responded when presented with extracted rut urine. When females were presented with samples of pentane, dichloromethane, and ethyl acetate extracted rut urine, the pentane extract significantly elicited wallowing behaviors in female moose. I wanted to determine which minor components collected from an active pentane extract that was separated by preparatory gas chromatography might contain the putative pheromone.

I hypothesized that when presented with 3 distinct fractions collected by preparatory gas chromatography females would respond to 1 of the fractions.

Experiment 1 tested the response of females to the Whole fraction (the effluent collected over the entire 30-minute period).

Experiment 2 tested the response of females to Fraction 1 (the effluent collected during the first 10 minutes).

Experiment 3 tested the response of females to Fraction 2 (the effluent collected during the second 10 minutes).

Experiment 4 tested the response of females to Fraction 3 (the effluent collected during the third 10 minutes).

The hypothesis that female moose would respond to Fraction 3 was unsupported. Female moose spent significantly more time engaging in Neutral behaviors than

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investigating the sample or the control. There may be several reasons to explain the lack of female response. One likely explanation is that the minor bioactive components remained on the column or had already eluted. Nonetheless, this experiment supports the hypotheses for Experiments 1, 2, and 3 -- the bioactive components were present in these Fractions.

For Experiments 1, 2, and 3 female moose spent significantly more time investigating the sample than the control. Additionally, these experiments elicited wallowing behaviors of females - a hallmark behavior exhibited by female moose when they gain access to a rutting pit. These data support a previous hypothesis (Section 4.3) that the active urinary constituents are retained after chemical extraction. To determine that the bioactive components of rut urine were present in the fractions collected, I compared GC/MS chromatographs of pentane extracted rut urine. Some of the minor, and all of the major peaks were present in the Whole, Fraction 1, and 2. Additionally, these urinary constituents have a low molecular weight, and are non-polar. Moreover, these data support a subsequent hypothesis (Section 4.6) that the more non-polar and low molecular weight substances typically elute first. Results from Experiments 1, 2, and 3 supported the hypothesis that when presented with 3 distinct fractions collected by preparatory gas chromatography females would respond to 1 of the fractions. Additionally, these data provided evidence that the putative pheromone(s) was present in either the Whole, Fraction 1, and/or Fraction 2. Moreover, these data indicate that the bioactive component(s) were present in a narrow window that was common to both Experiments 2, and 3 (Figure 3.13). Furthermore, the window is largely characterized by

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having a cyclic alkane and several C6-C12 unsaturated straight chain alcohols, which suggests that the chemosignal may be made up of multiple compounds.

4.6 Test of Bioactivity of 3α-Hydroxy-5β-androstan-17-one (steroid), and Flash Chromatography Eluants (Bioassays conducted in Autumn 2002)

Hypotheses for Experiments 1, 1a, 2, 3, and 4: Previous behavioral bioassays conducted indicated that female moose significantly responded to urinary constituents after chemical extraction. In particular, the 1999 behavioral bioassay (Section 3.3) established that the pentane extract significantly elicited behaviors (e.g., wallowing) when females were presented with rut urine. Because females responded to the pentane extract, I reviewed GC/MS data of the pentane extract to characterize the major bioactive constituents unique to rut urine. While reviewing those data, a steroid unique to the pentane extract urine was identified. Schwartz, et al. (1990) hypothesized that salivary steroids may play a role in synchronizing and inducing estrus in female moose. Based on female response to the pentane extract that contained the steroid, I wanted to test the hypothesis that females would respond to the steroid. To test this hypothesis, however, it was important that the steroid be presented in urine that did not have bioactivity (e.g., nonrut urine). Furthermore, the pH of nonrut urine was adjusted to that of rut urine. Adjustment of nonrut to the pH of rut urine was conducted to ensure substances in nonrut urine were in the same ionization state as found in rut urine.

Experiment 1 tested the response of females to pH-adjusted nonrut urine to which the steroid was added at the concentration (0.25 mg/ml) found in the pentane extracted rut urine.

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Experiment 1a tested female response to pH-adjusted nonrut urine to which the steroid concentration was increased 75-fold (18 mg/ml). To test for the efficacy, it is not uncommon to increase synthetic putative pheromone concentrations beyond that found in the actual sample (Rasmussen, et al., 1997).

Experiment 2 tested female response to *p*-cresol that was a major compound identified in rut urine, and the steroid to determine if the 2 compounds functioned synergistically. To test this hypothesis, *p*-cresol was added to the pH-adjusted nonrut urine, along with the steroid at a concentration of 18 mg/ml.

Experiment 3 tested the response of females to the steroid at a concentration of 18 mg/ml, and a positive control (rut urine).

Experiment 4 tested the response of females to the steroid in a solvent. To test this hypothesis, the steroid was dissolved in methanol to yield a concentration of 18 mg/ml.

Experiment 1: Steroid (concentration 0.25 mg/ml)

Although, the concentration of the steroid was near the concentration in pentane extracted rut urine it failed to elicit a significant response from females. In contrast to rut urine that has a strong odor, the steroid was odorless to the human nose. Two possible explanations for female lack of response are that: 1) females require it to be in a matrix (i.e., other compounds found in rut urine); and 2) the steroid has no pheromonal activity.

Experiment 1a: Steroid (concentration 18 mg/ml)

Although, the concentration of the steroid was increased 75-fold from that in the pentane extracted rut urine; the steroid failed to elicit a significant response from females.

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However, because it is unique to urine of rutting males, it may play an important role in the reproductive biology of moose.

Experiment 2: Steroid (concentration 18 mg/ml) and *p*-cresol (Cocktail)

Although, the concentration of the steroid was increased by 75-fold of that in pentane extracted rut urine; the Cocktail failed to elicit a significant response from females. In contrast to rut urine that has a strong odor, the steroid was odorless to the human nose. A possible explanation for female lack of response may be that the Cocktail has no pheromonal activity.

In conclusion, Experiment 1: Steroid (concentration 0.25 mg/ml), Experiment 1a: Steroid (concentration 18 mg/ml), Experiment 2: Steroid (concentration 18 mg/mg) and *p*-cresol (Cocktail), Experiment 3: Steroid (concentration 18 mg/ml) and positive control, and Experiment 4: Steroid (concentration 18 mg/ml) in methanol failed to elicit a significant response to those samples. Female moose spent significantly more time engaging in Neutral behaviors. The hypothesis that female moose would respond to the steroid was unsupported.

Hypothesis for Experiments 5, 6, and 7: Previous bioassays established that the bioactive constituents of rut urine are retained after chemical extraction. Furthermore, these urinary constituents are non-polar, and have a low molecular weight. Based on these chemical properties, I tested the hypothesis that female moose would respond to the eluants collected by flash chromatography on silica gel.

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Experiment 6: Flash Chromatography Fraction B3-11

This fraction was less complex than that of Experiment 5 (Figure 3.23). Therefore, it is likely that most but not all of the bioactive constituents eluted in the first fraction (Experiment 5). Typically, non-polar compounds elute the quickest when using silica gel. It is not uncommon that 100% of the compounds are recovered with flash chromatography; some bioactive constituents may have remained on the column, and were diluted into other fractions. In addition, there may have been evaporative loss of bioactive constituents during solvent removal. Females, however, spent more time investigating the sample than the control.

Experiments 7: Flash Chromatography Fractions B12-18 and C1-7

Most of the bioactive constituents likely eluted in the Fractions A13-17 (Experiment 5), and B3-11 (Experiment 6). Furthermore, this fraction was less complex than that of Experiment 5, and 6 (Figure 3.23).

In conclusion, the hypothesis that sufficient amounts of the bioactive urinary constituents were retained to elicit a significant response from female moose was unsupported for Experiments 6 (Fraction B3-11), and Experiment 7 (Fractions B12-18 and C1-7). These fractions either contained none of the bioactive urinary constituents or at levels too low to elicit a response from females.

Experiment 5 (Fraction A13-17) elicited a significant response to the sample, and support previous hypotheses that active urinary constituents are non-polar, and have a low molecular weight. Furthermore, these data support the hypothesis that a sufficient

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amount of the bioactive components were retained to elicit a response from female moose, and provided evidence that the putative pheromone was present in this fraction.

4.7 Evidence that Female Moose use the Main Olfactory System for Pheromone Detection

Olfaction is one of the principal sensory modalities for many animals, and chemosensory communication is well developed in most mammals (Stowers, et al., 2002; Trinh and Storm, 2003). Chemosignals are processed by two distinct olfactory systems: the main olfactory system (MOS), and the accessory olfactory system (AOS) (Dulac and Torello, 2003). Furthermore, each system has its unique pathway to different regions in the brain (Halpern, 1987) and terminates at the hypothalamus, which is responsible for behavioral (e.g., eating), and endocrine (e.g., changes in sex hormones) responses (Brennan, 2001). The AOS is believed to be involved mainly with matters related to reproduction (Halpern, 1987; Wysocki, 1989, Dulac and Torello, 2003). Furthermore, the receptor organ (vomeronasal organ) that is sequestered within the AOS processes chemosignals that may be linked to reproduction.

The presence of a well-developed vomeronasal organ (VNO) is often associated with a behavior termed 'flehmen'. Flehmen is common among ungulate species (Estes, 1972). Both males and females of some species exhibit flehmen in response to conspecific odors (Alteri and Müller-Schwarze, 1980; Reinhardt, 1983). During the mating season, male ungulates usually perform flehmen in response to chemosignals from females that may be important for coordinating reproduction (Alteri and Müller-Schwarze, 1980; Reinhardt, 1983; Crump et al., 1984).

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Both male and female moose possess a well-developed VNO (Clifford and Witmer, 2004). During the mating season, male moose usually display flehmen after sniffing the external genitalia or urine of females (Lent, 1974). During my studies, female moose did not flehmen to urine from the rut, or rut urinary constituents over the course of 50 hours of behavioral bioassays I conducted. These data indicate that the chemical cues in rut urine may be insufficient to elicit flehmen in female moose. These data further suggest that the MOS detects chemosignals important for reproduction.

4.8 General Conclusions with Future Directions

There are qualitative and quantitative chemical differences between the urine of adult male moose during the rut and nonrut periods. In this study, I was able to develop a behavioral bioassay that would aid in the identification of urinary constituents that elicited the behaviors observed in females during rut. Urine from the rut is a complex mixture that contains well over 100 compounds. Using a variety of chemical techniques (e.g., flash chromatography), I was able to delineate which partition of the urine had bioactivity (e.g., Prep GC – Section 3.5). Additionally, I used a variety of analytical tools (e.g., GC/MS) to characterize urinary constituents that may function as the putative pheromone(s), and eliminated some of the chemical differences that existed in rut urine that may not function as chemical signals (e.g., the Cocktail – Section 3.4). I have established behaviorally that female behaviors in response to urine from the rut are not elicited also by urine from periods outside the rut. Additionally, I have provided evidence that female moose may utilize the main olfactory system to detect chemosignals present in rut urine.

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This study established that the bioactive constituents are present in pentane extracted rut urine, and flash chromatography fractions. Further fractionation, synthesis, or purchase of these urinary constituents for presentation to female moose during behavioral bioassays may aid in the identification of male moose urinary pheromone(s).

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